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Special Notice

to Contributors and Subscribers

Beginning with the February 1960 issue of the Journal of Cellular and Comparative Physiology, the publication schedule of the Journal will be on time and our aim is to keep it that way.

Contributors will be particularly interested in the additional aim this on-time publication schedule makes possible, namely, the issuance of papers within three months of acceptance. Even more rapid publication will be available for short, complete articles contributed to the section *Comments and Communications.*

The double column format, started in the August 1959 issue has been one of the principle factors in making this prompt publication possible.

(Contributors will find additional information on the inner, back cover.)

EDMUND NEWTON HARVEY

1887–1959

The Board of Editors of the Journal of Cellular and Comparative Physiology and The Wistar Institute Press record with a deep sense of loss the death of Dr. Edmund Newton Harvey on July 21, 1959.

During 1930 and 1931 Dr. Harvey's keen interest and drive led to the establishment and publication of the Journal. He, with a few enthusiastic friends, saw the need for a new periodical in the area of General Physiology. It was he, who, after numerous discussions with fellow physiologists, made the decision to name it the Journal of Cellular and Comparative Physiology. From the first issue in February of 1932 to the thirteenth volume in 1939 he served as its Managing Editor, and on the Editorial Board until his death. During his tenure as Managing Editor the Journal developed a broad coverage of the area, achieved quality and distinction.

Prehemolytic Studies of Photosensitized Rabbit Erythrocytes

J. W. GREEN, H. F. BLUM[1] AND A. K. PARPART
Rutgers University, New Brunswick, N. J., National Cancer Institute, Bethesda, Md.[2] and Princeton University, Princeton, N. J.

Rose bengal, the dye used in this study, photosensitizes red blood cells so that they are hemolyzed when exposed to light (photodynamic action). In sufficiently high concentration the dye may bring about the hemolysis of cells in the absence of light (Dunkelwirkung). The underlying reaction is different in the two cases, the reaction in the dark being independent of O_2, the light reaction O_2-dependent. The light reaction involves repeated participation of the dye molecules, which remain unaltered at the end of the reaction, and hence it is possible to use such low dye concentrations that the dark reaction is negligible. On the other hand if sufficiently high concentration of dye is used, the light reaction may be negligible because the light is virtually all absorbed by the outermost layers of the system. Thus it is possible to study the two types of reactions separately. This picture is paralleled with many dyes and pigments and in a variety of living systems (for a general discussion see Blum, '41).

Whereas there have been many studies of hemolysis by photosensitizing dyes, few have been concerned with the prehemolytic changes which are considered in the present paper. Davson and Ponder ('40) found a marked loss in potassium from rabbit erythrocytes photosensitized with rose bengal, and briefly exposed to light, when these cells were subsequently incubated in the dark. Prehemolytic volume increases in ox and sheep red cells photosensitized with other fluorescein dyes were found by Koch, Liechti and Wilbrandt ('46); who attributed these increases in volume to changes in permeability to salts.

The present study explores the sodium and potassium exchanges, and glucose usage, in rabbit cells prior to hemolysis after various treatments with rose bengal.[3]

METHOD

Erythrocytes were obtained from rabbit blood withdrawn by heart puncture and defibrinated. The red cells were separated by centrifugation, washed twice with isotonic phosphate buffered NaCl at pH 7.0 and made up to dilute suspensions as follows: Packed cells 2.5 to 5.0 ml/l plus 5.55 M/l glucose, added to 30% 0.11 M phosphate buffer at pH 7.0, 70% 0.16 M NaCl. The concentration of rose bengal in the suspending medium was 2×10^{-6} M or less; such dye concentrations do not produce appreciable hemolysis in the dark.

For irradiation the sample of red cell suspension was contained in the space between two concentric cylindrical glass tubes 5 mm apart. Inside the inner tube was a 15 watt tubular "daylite" fluorescent lamp, the intervening space being filled with running water at approximately 15°C. This apparatus permits the cells to be irradiated under conditions of constant temperature, in a thin layer so as to reduce optical filter effects. Two-hundred-milliliter samples of the cell suspensions were irradiated in this apparatus for very short periods, usually 30 seconds. Such dosage produced little hemolysis even after long periods of incubation following the irradiation.

Irradiated and control samples were centrifuged to concentrate the cells, and supernatant withdrawn. Mixtures of supernatant and packed cells were reconstituted to give suspensions of 5.0% cells; these

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare.

[3] A preliminary report appeared in this journal ('55).

TABLE 1

Effect of rose bengal on hemolysis and cation exchange in rabbit red cells

Treatment	% Hemolysis	Cations	
		K	Na
		Meq/l cells	
Cells without RB exposed to light for 4 min. and then incubated in dark	5.2 at 150 min.	100.3	32.0
Cells with RB exposed to light for 4 min. and then incubated in dark	24.1 at 30 min.	5.9	136.0

were incubated at 37°C. All procedures after preparation of the dilute suspensions were carried out in a darkened room. For incubation the cell suspensions were placed in 50-ml pyrex bottles blackened on the outside, carried on slanted rollers which were in motion 15 minutes out of each 30-minute period during the 6 to 9 hours of incubation. Aliquots of cells and of whole suspensions were taken for analysis, initially, midway, and at the end of the incubation period. The cells were analyzed for Na and K by flame photometry using cell hemoglobin concentration (determined spectrometrically after hemolyzing the cells) as a measure of volume. Whole suspensions were analyzed for glucose content by the method of Nelson ('44) and Somogyi ('52). The supernatants were analyzed spectrophotometrically for hemoglobin to determine the amount of hemolysis that had occurred. Some hemolysis occurred in all cases as might be expected after the long incubation period at 37°C, but since the cation values were determined on unhemolyzed cells the differences observed represent prehemolytic changes in those cells. In most cases less than 10% of the cells hemolyzed.

RESULTS

Prehemolytic effects of photosensitization. Initial experiments were performed to establish that the prehemolytic changes observed in red cells photosensitized by rose bengal were to be attributed to photodynamic action. While hemolytic changes have been extensively described (see Blum, '41), Davson and Ponder ('40) were the first to note an acceleration in K loss in photosensitized cells prior to hemolysis. Table 1 presents the results of an experiment designed to show that in photosensitized red cells hemolysis is preceded by Na and K exchanges. The table show that the rose bengal photosensitized cell after an incubation period only one-fifth a long as that of the control cells have ex changed Na and K to nearly diffusion equi librium. This marked acceleration in ca tion exchange is accompanied by a 5 fold increase in the amount of hemolysi in the photosensitized cells over that ob served in the cell suspensions without dye

Oxygen dependence of prehemolyti effect. The oxygen dependence of the ca tion exchange is indicated in table 2. It i

TABLE 2

Oxygen dependence of rose bengal effect in rabbit red cells

Treatment	% Hemolysis	Cations	
		K	Na
		Meq/l cells	
With nitrogen	5.8	91.3	33.9
With air	8.7	70.0	55.7

seen that in an atmosphere of nitrogen, the cation exchanges of photosensitized cells are much less than in air, as is also the case for the amounts of hemolysis tha are recorded in the table. There seems little question that the photochemical re action underlying the increased cation ex change is a photosensitized oxidation by molecular oxygen, as is well known for hemolysis.[4] It seems likely that the photo chemical changes underlying the increased cation exchange are identical with those underlying hemolysis, both resulting from increase in permeability of the cell mem brane.

Reciprocal exchange of cations. Figure 1 illustrates the course of the net cation

[4] The complete O_2 dependence of photosensi tized hemolysis is indicated by recent studies o Cook and Blum ('59).

exchanges, based on determinations of sodium and potassium made at the beginning of the incubation period after 4 hours and after 8 hours of incubation. The data are arranged in the figure so as to show the reciprocal exchange of K^+ and Na^+, the movement of K out of the cells being accompanied by movement of Na into the cells although the proportionality is not constant. Cell suspensions containing 10^{-6} M rose bengal but not exposed to light, show only slight, linear cation exchange during the 8 hours of incubation. This is closely paralleled in control suspensions containing no dye where the change is likewise linear as found by Parpart and Green ('53). There is thus no detectable dark effect of the dye at this concentration. On the other hand, figure 1 shows that rose bengal containing suspensions, which had been exposed to light for 30 seconds, displayed marked, progressive, cation exchange. It thus seems clear that the increase in cation exchange is to be attributed entirely to photochemical reaction.

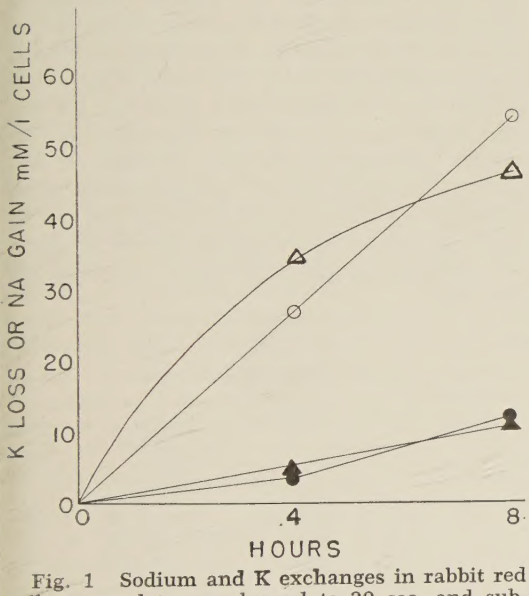

Fig. 1 Sodium and K exchanges in rabbit red cells exposed to rose bengal to 30 sec .and subsequently incubated at 37°C. ▲ = K, cells not photosensitized; △ = K, cells photosensitized; ● = Na, cells not photosensitized; ○ = Na, cells photosensitized.

Glucose usage and cation exchange. In table 3, cation exchange is compared with glucose usage in averaged data from several experiments. It is seen that glucose usage is slightly increased by photosensitization but that the magnitude of the change is small compared to that of the cation exchange. Similar results have been obtained in other experiments (e.g., see table 4), and it seems clear that whatever the cause of the increased glucose usage, it is not closely related to the amount of cation exchange.

A possible explanation of the increased glucose disappearance is that the increase in permeability brought about by the photochemical reaction permits glucose to diffuse into the cell at a greater rate than at higher or lower pH values (Laris, '58, Faust and Parpart, '59). There is no direct evidence that photosensitized oxidization interferes with glycolysis, although this cannot altogether be ruled out, and at least a small amount of enzyme inactivation might be expected.

The reduction in glucose use in rose bengal treated cells without light shown by table 3 at pH 7.0 has also been shown by some recent work in one of our laboratories (J. W. Green), to occur at pH 7.4 in both dye treated photosensitized and nonphotosensitized cells. The reduction in glucose disappearance can be overcome by increasing the external glucose concentration.

Effect of hydrogen ion concentration. Table 4 shows the effect of hydrogen ion

TABLE 3

Effect of rose bengal on cation exchange and glucose use in rabbit red cells

Rose bengal	Illumination period	Average cation values		Na/K	Glucose used
		K loss	Na gain		
M/l	*seconds*	*mMoles/l cells/hr.*			*mMoles/l cells/hr.*
0	0	1.5	1.6	1.07	0.120
10^{-6}	0	1.8	1.9	1.06	0.109
10^{-6}	30	7.4	8.1	1.08	0.138

concentration on cation exchange and glucose use. Over the range studied the effect of pH on glucose use is small compared to its effect on cation exchange. In rose bengal photosensitized cell suspensions glucose use was highest at pH 7.38, intermediate at 7.17 and lowest at 7.0, a finding in agreement with the data on another hemolytic system, Parpart and Green ('53). Potassium loss was highest at pH 7.0 and lowest at 7.38 in photosensitized red cells and inconsistent for cells in the dark. Sodium gain was highest at pH 7.38 and lower but at nearly equal levels at pH 7.14 and 7.0. No explanation is given these findings. It is of interest however, that photosensitized rabbit red cells at pH 7.38 exhibit a Na/K exchange ratio very similar to human red cells at the same pH but nonphotosensitized (Harris, '54).

Dye concentration and inner filter effect. The amount of photosensitized oxidation of this type should be directly proportional to the amount of light absorbed by the dye, but otherwise independent of the dye concentration. However, under most conditions of exposure the nonparticipating dye acts as an optical filter, so that the amount of reaction is observed to fall off with increasing dye concentration. Blum and Gilbert ('40) were able to minimize the inner filter effect by exposing the cells directly to the light without intervening dye solution, and in this case found that over a wide range of dye concentration the threshold for hemolysis was directly proportional to the light absorbed by the dye. In the present case the conditions are less favorable, since there may be as much as 5 mm of dye solution between a given cell and the plane of incidence of the light, and a certain amount of filtering by the stained cells themselves is to be expected. Table 5 shows the effect of rose bengal concentration in cation exchange and glucose use under these condtions of illumination. The dye concentration and the time of exposure were varied reciprocally so that the same amount of cation exchange should have occurred in all the illuminated samples if the same fraction of the incident light reached the cells. It is noted that this is the case, within limits of error, in the two lower concentrations, but that in the higher concentrations the amount of cation exchange falls off progressively with concentration, as would be expected if the dye was acting as an inner filter to reduce the amount of photochemical reaction on the cells.

TABLE 4

Effect of hydrogen ion concentration on cation exchange and glucose usage

Rose bengal	Illumination period	pH	Rates of cation exchange		Glucose used
			K loss	Na gain	
M/l	*seconds*		*mMoles/l cells/hr.*		*mMoles/l cells/hr.*
10^{-6}	0	7.0 –6.98	2.99	0.47	0.068
10^{-6}	30	7.0 –6.98	7.13	3.82	0.089
10^{-6}	0	7.17–7.14	3.17	1.71	0.093
10^{-6}	30	7.17–7.14	6.11	3.56	0.106
10^{-6}	0	7.38–7.32	1.81	1.93	0.077
10^{-6}	30	7.38–7.32	5.28	7.34	0.116

TABLE 5

Effect of rose bengal on cation exchange and glucose utilization when period of illumination is increased and dye concentration decreased reciprocally

Rose bengal	Illumination period	Average cation values		Na/K	Glucose used
		K loss	Na gain		
M/l	*seconds*	*mMoles/l cells/hr.*			*mMoles/l cells/hr.*
2×10^{-6}	0	1.7	2.4	1.41	0.196
1×10^{-6}	30	6.4	8.2	1.28	0.215
5×10^{-7}	60	7.5	11.7	1.56	0.220
2.5×10^{-7}	120	7.5	12.5	1.57	0.246

DISCUSSION

Rabbit red cells exposed to rose bengal and light take up Na and lose K at much more rapid rates than do nonphotosensitized cells. Since in a nitrogen atmosphere dye treated cells exposed to light exchange cations at the same rate as nonphotosensitized cells, the evidence indicates that the accelerated cation exchange in the photosensitized cells is a result of photochemical action.

The conditions of these experiments produced only moderate volume changes in the cells and yielded, in photosensitized cells, net K losses of 6% per hour of initial cellular K levels and net Na gains only slightly less. Usually after 6 hours the rate of K loss is reduced while Na gain continues with little diminution until hemolysis occurs (fig. 1). Sodium and K exchange in the controls are linear over the 8-hour period as was found by Parpart and Green ('53). When the period of illumination is reduced, nearly equal exchange of Na and K occurs in photosensitized cells. With increased time of illumination or increase in dye concentration the exchange becomes more unequal, Na entering more rapidly than K leaves the cells. The cation exchanges observed here are net fluxes and represent for K the difference between an influx associated primarily with cells metabolism and an efflux attributed to diffusion, while the Na movements are the converse of the K.

There is general agreement that glycolysis is intimately associated with the maintenance of mammalian red cell Na and K concentrations over a range of *in vitro* experimental conditions. No corresponding recognition exists of those experimental procedures by which increased net Na and K fluxes occur without any apparent interference with glycolysis. The data of table 3 show that with photosensitization a greater amount of glucose disappears than in control suspensions while the net fluxes are markedly increased. Information is lacking about the use of this extra glucose, since lactate formation, with one or two exceptions, was not measured. Where measured less lactate was produced in photosensitized than in control suspensions. However, from the glucose data alone, it would seem that those ion exchanges, K influx and Na efflux, usually associated with glycolysis are not interfered with by rose bengal photosensitization under the experimental conditions used here.

While we have no certain knowledge of the manner in which an external hydrogen ion concentation affects either cell metabolism or the cell surface of red cells, studies on the storage survival of human red cells, (Loutit, '45 and Parpart et al., '47) have shown the importance of this ion in the external medium. Parpart and Green ('53) working with rabbit red cells reported that in the presence of glucose these cells lost K at approximately the same rates at pH 7.5 and 7.0 and at a more rapid rate at pH 6.5. Glucose use, by contrast, appeared to occur most rapidly at pH 7.5 and decreased to pH 6.5.

SUMMARY

Prehemolytic changes in photosensitized rabbit red cells were studied. Washed cells, maintained in phosphate-buffered NaCl solutions which contained very low concentrations of rose bengal were exposed for brief periods to visible light in air. The cells were subsequently incubated in the dark at 37°C and Na-K exchange and glucose disappearance observed with time.

Brief periods of photosensitization markedly accelerate Na uptake and K loss while only slightly increasing the rate of glucose disappearance. Initially the cation exchange is equivalent but generally after 6 hours Na enters the cells more rapidly than K leaves. The effect of dye concentration, of length of illumination and of hydrogen ion concentration were observed.

It appears likely that the acceleration of cation exchange with photosensitization is the result of cell surface alteration rather than to an effect on cell metabolism.

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Histochemical and Biochemical Studies of Liver Glycogen in a Hibernator, Myotis lucifugus lucifugus[1]

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INTRODUCTION

Many studies have been made concerning the carbohydrate metabolism of hibernating mammals. These have been ably reviewed by Suomalainen ('35), Kayser ('50), and Lyman and Chatfield ('55). The object of much of this research has been to determine the variation in the quantity of glycogen and blood sugar in the torpid animal. Unfortunately, the results from these early experiments are not all in accord, even when the same species of animal is used. One group of workers have observed an accumulation of glycogen in the early part of hibernation, followed by a decrease in the amount of glycogen throughout the remainder of hibernation (Bernard, 1859; Aeby, 1875; Luchsinger, 1875; Voit, 1878; Dubois, 1896; Rulot, '02; Weinland and Riehl, '08). Others have found that little glycogen accumulated and that glycogen levels remained relatively constant during hibernation (Valentin, 1857; Kulz, 1881; Reach, '10). Some of these works have been followed by the conflicting conclusions that: the hibernating animal maintains or increases its glycogen stores by glyconeogenesis (see Rulot, '02; Atkinson, '22) and, that there is no evidence for the formation of glycogen from non-carbohydrate sources (Valentin, 1857; Schiff, 1859; Kulz, 1881).

While there has been an appreciable variability of results and interpretations in glycogen studies of animals in hibernation, the work on the active (awake) and transitional periods (leaving and entering hibernation) has been in close accord. It is generally agreed that liver glycogen is lowered when used as a source of energy during the vigorous process of waking from hibernation (Bernard, 1859; Weinland and Riehl, '08; Ferdmann and Feinschmidt, '32; Lyman and Leduc, '53). Seasonal changes in the liver glycogen levels of hibernating animals are accompanied by changes in blood sugar levels. Most reports agree that blood sugar levels are higher in the active animal than in the torpid animal (Dubois, 1896; Endres, '30; Dische, '31; Ferdmann and Feinschmidt, '32; Stucky et al., '42; Suomalainen, '43; Woodward and Condrin, '45; Dodgen and Blood, '56).

It appears that many of the conflicting results and interpretations concerning liver glycogen levels of hibernating animals, are the result of an insufficient amount of experimental data, for only one, or a few scattered periods of the hibernator's yearly activity have been represented in most of these determinations. As a consequence of this lack of experimental data, the histochemical and biochemical determinations of liver glycogen over a yearly period were undertaken. The primary purpose of this study was to make comparisons between liver glycogen levels and distribution during the different stages of activity of the yearly cycle. Hibernating animals, also, were subjected to experimentally altered temperatures and adrenalectomy for the purpose of throwing some light on the question concerning the ability of the hibernating animal to form glycogen from non-carbohydrate sources. The bat, *Myotis lucifugus lucifugus*, was used for this study because it was easy to maintain in hibernation, it was quite attainable throughout the year, and it does not feed during the hibernating period.

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[1] Partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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The results of these determinations will show a general decrease in liver glycogen from extremely high levels at the onset of hibernation to extremely low levels at the end of the active period. Evidence is presented which suggests that the hibernating bat is capable of partially restoring liver glycogen levels which were previously lowered when hibernating animals were subjected to increased environmental temperatures. In those instances where no uniform distribution of glycogen is found in the hepatic lobule, there is always more glycogen in the peripheral zone than in the central zone of the hepatic lobule. Blood sugar levels were determined whenever it was felt that a check was needed on the liver glycogen picture.

MATERIALS AND METHODS

This study is based on the histochemical and biochemical analysis of liver glycogen in mature female bats (Myotis lucifugus lucifugus) of the family Vespertilionidae. Female bats were used because they were the easiest to obtain throughout the year, and the use of one sex precluded any variations that might be due to the sexual cycle (e.g., as noted in mice by Deane, '44). The adults of this species have an average length of about 89 mm and their body weight varies from 5 to 9 grams.

Liver glycogen values of hibernating bats were obtained from animals collected during the winter on monthly trips to caves in New York, New Jersey and Pennsylvania. These animals were maintained in hibernation in a 27″ × 42″ × 45″ artificial cave at 5° ± 2°C for periods not exceeding one month. Although water was available to all of the animals, no food was given to them at any time. The "artificial cave," hereafter called the cold-room, was kept dark and humid, and every attempt was made to keep the animals from being disturbed.

All bats in the summer colony were collected in the afternoon and maintained without food at room temperature (25° ± 3°C) until the following morning when they were sacrificed. Fasted animals from the summer colony were desired for two reasons: first, they were to be compared with hibernating bats which normally fast and, second, fasting would eliminate many changes that might be produced by a diurnal cycle (as noted in mice by Deane, '44; and in rats by Deuel et al., '38; and Pitts, '43).

In order to acquire enough liver from each bat for biochemical and histochemical procedures, it was necessary to sacrifice the animal. Hibernating bats were killed by lowering their heads into a solution of dry ice and acetone, since preliminary investigations showed that hibernating bats killed by lowering their body temperature rapidly below the lethal level exhibited far less muscular activity than those killed by decapitation, ether, nembutal, or a blow on the head. Animals from the summer colony likewise were killed in this manner.

When blood sugar and liver glycogen were determined simultaneously, it was necessary to kill the hibernating animals with ether, since hibernating bats killed by other means had such a sluggish blood flow it was impossible to obtain the necessary 0.1 ml of blood. These hibernating bats were removed from the cold-room immediately before they were killed. Although blood could be obtained easily from summer bats which had not been killed by ether, the same procedure was used so that variations in results could not be attributed to a difference in methods.

Immediately after the bats were killed, they were weighed and the entire liver was removed from each animal. Two small pieces of each liver were fixed for histochemical determinations; the remainder was divided into two parts of about 70–90 mg each for biochemical determinations.

The method of glycogen separation from the tissue was essentially that described by Good, Kramer and Somogyi ('31). After the liver was digested in 2.0 ml KOH, glycogen was precipitated with 1½ volumes of alcohol, centrifuged and dissolved in water. Aliquots were taken so that the amount of glycogen to be determined would range from 50 to 125 gamma of glucose equivalents. Glycogen was determined by the anthrone method (Morris, '48; Seifter et al., '50) and the resulting solution was read with the 660 mμ filter on the Klett-Summerson colorimeter. The glycogen in the liver is reported as glucose in grams per cent.

The liver obtained for microscopic study was fixed in chilled Rossman's fluid (Rossman, '40) for 9–10 hours, dehydrated in absolute alcohol, cleared in chloroform and imbedded in 56° — 58°C tissuemat (Fischer) in a vacuum oven. Sections were cut at 6 μ and stained for glycogen by the periodic acid Schiff method of McManus ('48). Similar sections were digested with saliva and treated by the above methods to check the specificity of the reaction.

After bats were killed by ether, they were weighed, an incision was made in the ventral wall of the thorax, and the ventricular portion of the heart was snipped off. Mixed blood was then removed by a 0.1 ml Folin-Malmros pipette and precipitated in 10 ml of a dilute tungstic acid solution. Blood sugars were determined colorimetrically by the Folin-Malmros method ('29). The resulting Prussian blue solution was read with the 540 mμ filter on the Klett-Summerson colorimeter. The blood sugars are reported in mg%. After the blood was removed, liver was taken by the same procedure described for the animals killed by dry ice and acetone.

In the study of "normal" bats over a yearly period, animals were killed by dry ice and acetone in every month from July, 1953 to July, 1954. Over-lapping determinations were run in August, 1954 and February, 1956. The liver glycogen levels of hibernating bats were determined from October, 1953 through May, 1954, and the liver glycogen levels of bats from the summer colony were determined in the months of July, 1953 through September, 1953, and in June of 1954.[3] Blood sugars were determined from February through June. All of the animals used were killed between 7:00 A.M. and 9:00 A.M. on the day determinations were made.

In one series of experiments, 6 hibernating bats were removed from the cold-room and maintained without food at room temperature (25° ± 3°C) for 36 hours. At the end of this period of time, three of the animals were killed by dry ice and acetone. The other three were returned to the cold-room for 72 hours, after which time they were killed by the same method as the three that were not returned to the cold-room. Blood sugar determinations were performed on several ether-killed animals, which were subjected to these temperature conditions.

In another series of experiments hibernating bats were bilaterally adrenalectomized. Control animals were bilaterally laparotomized. Before each group of operations, bats were removed from the cold-room and brought to activity at room temperature. Each active animal was anesthetized with ether and secured to an operating board. Two dorsal incisions were made in the lumbar region and the adrenals were removed with an electric cautery needle. The wounds were then closed by surgical silk. Each operation took about 40 minutes.

As soon as the adrenalectomized and laparotomized bats had come out of anesthesia, most of them were returned to the cold-room for 42–136 hours before they were killed. Of three small groups of animals, one group was maintained at room temperature for 20 hours and then returned to the cold-room for 72 hours, a second group was returned to the cold-room for 92 hours and then put at room temperature for 12 hours and, a third group was maintained at room temperature for 15 hours before the animals were killed. All animals were offered only physiological saline for drinking purposes. All of these animals were killed by ether.

RESULTS

A large standard deviation was observed in practically all biochemical data of liver glycogen and blood sugar in the various groups of bats studied. It is possible that this large deviation is correlated with extreme variations in the muscular activity of animals in these groups, since some bats may have been more active, or inactive, immediately before determinations of liver glycogen and blood sugar were made

[3] Since the bats used in May were the last ones collected in caves, it is quite probable that some of these animals had aroused from hibernation and made several trips out of the cave before they were collected (Wimsatt, '44). All of the bats, however, were collected deep in the cave, appeared to be hibernating, and were maintained in this state in the artificial cave. For these reasons this group of animals was considered generally to be representative of the last month of hibernation.

TABLE 1

Average monthly tissue carbohydrate levels in normal bats from July, 1953 through June, 1954

Month	Condition	Dry ice and acetone killed			Ether killed			
		No. animals	Body weight	Liver glycogen	No. animals	Body weight	Liver glycogen	Blood sugar
			gm	*gm %*		*gm*	*gm %*	*mg %*
July	Active	9	7.5	0.815 S.D.,0.776				
August	Active	11	6.6	0.477 S.D.,0.260				
Sept.	Active	4	8.4	0.176 S.D.,0.155				
Oct.	Hibernating	6	8.0	4.35 S.D.,1.64				
Nov.	Hibernating	6	7.8	3.82 S.D.,1.62				
Dec.	Hibernating	6	7.4	2.82 S.D.,0.794				
Jan.	Hibernating	3	6.8	2.78 S.D.,1.72				
Feb.	Hibernating	6	7.8	4.13 S.D.,1.08	5	5.9	2.32 S.D.,1.32	135.7 S.D.,68.42
March	Hibernating	16	6.4	2.20 S.D.,1.03	1	6.2	0.308	274.5
April	Hibernating	8	6.7	2.51 S.D.,1.21	5	6.5	1.34 S.D.,1.03	121.6 S.D.,77.07
May	Hibernating	12	5.2	1.41 S.D.,1.20	4	5.3	1.12 S.D.,1.17	167.7 S.D.,87.74
June	Active	14	8.1	0.445 S.D.,0.243	4	6.9	0.520 S.D.,0.327	158.6 S.D.,45.48

than the other animals in each of the groups.

Quantitative determinations in bats killed by dry ice and acetone

The results of the biochemical determinations of liver glycogen in the bat, *Myotis lucifugus lucifugus*, are summarized in the form of monthly mean liver glycogen levels in table 1. It is noted that the mean liver glycogen level is the highest in those bats which were killed in October, the first month of hibernation, and that generally there is a progressive monthly decrease in liver glycogen in bats from October to the following September.

With the exception of the February and April animals, liver glycogen in bats dropped progressively by 68% from the first month of hibernation (October) to the last month of hibernation (May). Although the slight but not statistically significant increase in liver glycogen in April bats apparently was due to individual variation, the extremely high liver glycogen level of the February animals could not be similarly explained. The fact that the February bats were collected from an entirely different location (Hibernia, New Jersey), with possible different environmental conditions, than the other hibernating animals in this study, appeared to be significant in explaining these high liver glycogen levels. In order to verify this, bats were collected from both habitats in February, 1956. The results of these simultaneous liver glycogen determinations showed that the mean liver glycogen level of hibernating bats from New Jersey was considerably greater than that of the Pennsylvania bats, and that the mean glycogen level of the latter group was similar to that of the January animals (fig. 1).

The mean liver glycogen levels in bats from the summer months (June through September) were all significantly lower than the mean values of the hibernating bats. The low quantity of liver glycogen in June bats was the one exception to the progressive decrease in liver glycogen during the summer. The fact that 8 of the 14 June bats were pregnant should be taken into consideration, for the mean liver glycogen of the pregnant animals was 38% less than that of the non-pregnant animals. Less glycogen in pregnant animals than in non-pregnant animals has been reported by Kosterlitz and Campbell ('47) in their work on the rat.

In overlapping determinations of August, 1954, the mean liver glycogen level

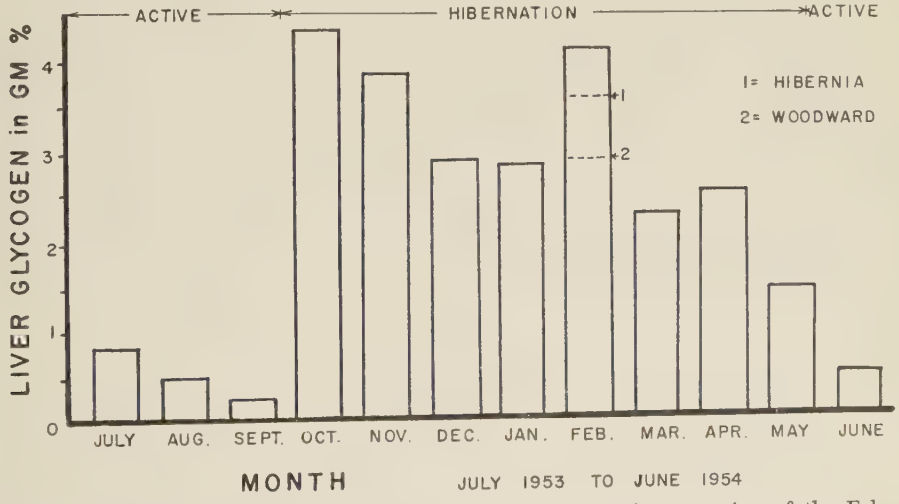

Fig. 1 Liver glycogen in normal bats. All animals, with the exception of the February group, were collected in New York and Pennsylvania. The February bar represents the liver glycogen level of bats taken from Hibernia, New Jersey in 1954. The dotted lines stand for levels of liver glycogen in animals collected at Hibernia, New Jersey and Woodward, Pennsylvania in February, 1956.

(0.469 gm %) was very similar to that of August, 1953 (0.477 gm %).

Histochemical determinations in bats killed by dry ice and acetone

In the first month of hibernation, October, glycogen was distributed uniformly throughout the lobules of all bats and the cytoplasm of the hepatic cells was observed to be heavily laden with glycogen (fig. 3). As is to be expected, there was no glycogen in the hepatic cells of the saliva-control sections (fig. 4).

From November through February there was a uniform glycogen distribution in the hepatic lobules of most bats. In each of these months, the bat with the least glycogen was observed to have a very small and almost indistinguishable decrease in glycogen in the central zone of the hepatic lobule (fig. 5).

In the last three months of hibernation (March, April and May), fewer bats had a uniform distribution of liver glycogen. In both March and April bats, 38% showed a gradual decrease in glycogen toward the central zone of the hepatic lobule, whereas over 58% of the May animals showed this zonation (fig. 6). No glycogen, or only occasional minute granules, was found in two of the 12 animals of the May group. Thus, it appears that liver glycogen decreases as hibernation progresses and that this decrease is more evident in the central zone than in the peripheral zone of the hepatic lobule.

In June, July and August, a sharp peripheral zonation of glycogen was found in all bats with more than 0.2 gm % of glycogen (fig. 7). In the rest of these bats and in all September animals, only traces of glycogen were found in a few cells of the middle and peripheral zones of the hepatic lobules (fig. 8). The distribution of liver glycogen in pregnant bats was similar to that of non-pregnant bats with comparable amounts of glycogen.

Body weight in normal bats killed by dry ice and acetone

A striking month-to-month correlation is found between the quantity of liver glycogen and the body weight of hibernating bats (fig. 1 and fig. 2). With the exception of the February and April determinations, there was a continuous decrease in the body weight of bats during hibernation. This decrease is compatible with the observations on bats of Rulot ('02), Reach ('10) and Beer and Richards ('56). The fact that both body weight and liver glycogen levels of February bats from New

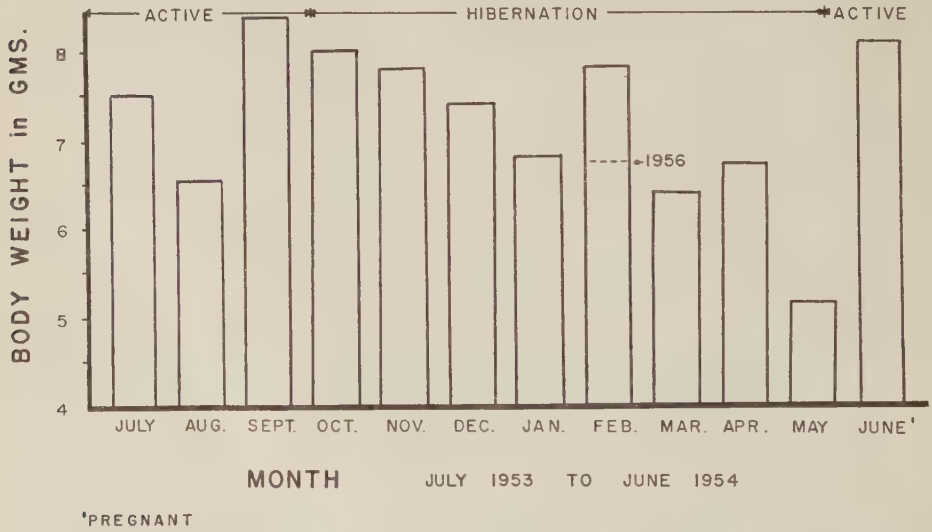

Fig. 2 Body weights in normal bats. The dotted line in the February bar represents the identical average body weights of the Hibernia, New Jersey and the Woodward, Pennsylvania groups of animals collected in 1956.

ersey are higher than those of the Pennylvania animals from the preceding nonth, suggests basic differences in the ibernating habits of bats collected from lifferent regions. This suggestion is supported by the comparison in February, 956 which showed more liver glycogen in New Jersey bats than in Pennsylvania ats (fig. 1) even though animals had een selected so that the average body veight of each of these groups was essenially the same as the average body weight f animals from the preceding month fig. 2). If environmental temperature lays a relatively strict role in the regulaion of the advent and duration of hibernaion in these animals, then climatological lata from the U. S. Department of Commerce Weather Bureau (which showed hat frost occurred 23 days earlier and mean annual temperatures were 3° to 3.5°F lower in the Woodward area) support a view that the Hibernia bats possibly ntered hibernation at a later date than he Woodward animals. In this case the February month was an earlier period in he hibernation of the Hibernia bats than n the Woodward bats.

There is a less-striking correlation of iver glycogen with body weights in animals from the summer colony. The June body weight level (fig. 2) would be less 7.17 gm) and would correspond with the verage weights recorded for the July and August animals if only the non-pregnant animals were considered. An increase in body weight of 21% and observed increases of subcutaneous, mesenteric, and brown fat were found in bats from August to September. An increase in brown fat for this species during this period has been observed previously by Rémillard ('58).

Quantitative and histochemical determinations of liver glycogen in bats killed by ether

A comparison of liver glycogen levels in table 1 indicates that the ether-killed animals from the months of February through May had lower glycogen levels than the dry ice and acetone-killed bats from the same months. Since ether killing always resulted in a preliminary arousal of the hibernating bat, the data suggest that ether activates hibernating bats and lowers liver glycogen levels. This is supported indirectly by the facts that the ether-killing of active June bats did not result in lowered liver glycogen values and, that the inconclusive blood sugar levels appeared to be indicative of variances in activity. Histochemically, the patterns of glycogen distribution observed in ether-killed bats were similar to those found in dry ice and acetone killed animals with the same liver glycogen levels. The numerous cases of glycogen zonation all were characterized by a greater concentration of glycogen in the peripheral zone of the hepatic lobule than in the central zone.

Liver glycogen in hibernating bats subjected to experimentally altered environmental temperatures

It was observed that all of the animals placed at room temperature were maintained in an aroused and active state for 36 hours and, that those returned to the cold-room for 72 hours reassumed the torpid state of hibernation. The quantity of liver glycogen in the animals killed at room temperature is observed to be significantly lower than that of the normal hibernating bats of the comparable month of hibernation (table 2). Also, it is observed that a

TABLE 2

Liver glycogen in hibernating bats subjected to experimentally altered environmental temperatures

Condition	No. animals	Liver glycogen	S.D.
		gm %	*gm %*
At room temperature for 36 hours	3	0.193	0.147
At room temperature for 36 hours then returned to the cold room for 72 hours	3	2.76	1.24
Undisturbed, normal, hibernating in cold room	3	2.78	1.72

TABLE 3

Liver glycogen and blood sugar levels of adrenalectomized and laparotomized bats

Postoperative conditions	Adrenalectomized			Laparotomized		
	No. animals	Liver glycogen	Blood sugar	No. animals	Liver glycogen	Blood sugar
		gm %	*mg %*		*gm %*	*mg %*
Return to cold-room until sacrificed	8	0.571 S.D.,0.542	32.67 S.D.,6.80	4	2.11 S.D.,0.865	92.13 S.D.,39.61
Room temperature for 20 hours then cold-room for 72 hours	3	0.094 S.D.,0.041	30.20 S.D.,0.00	1	2.61	57.00
Cold-room for 92 hours then room temperature for 12 hours	2	0.026 S.D.,0.018	22.27 S.D.,3.61	1	1.02	67.58
Room temperature for 15 hours	3	0.033 S.D.,0.018	21.57 S.D.,4.77	1	1.13	206.7

tatistically significant difference in the quantity of liver glycogen is found between hose animals killed at room temperature nd those returned to the cold-room, with he mean glycogen values of the latter roup (2.76 gm %) almost the same as hat of the normal bats from the corresponding month of hibernation (2.78 gm %). Thus, it appears that liver glycogen decreases greatly when bats are aroused rom hibernation and, that liver glycogen evels appear to be partially restored when animals are returned to the torpid state of ibernation.

All animals subjected to these same temperature differences and killed by ether for blood sugar determinations had only low iver glycogen levels, appeared to be aroused from hibernation, and showed much movement upon ether administration. Thus, it is believed that true blood sugar levels for the hibernating bats in his study are not available.

The histochemical preparations supported the biochemical results and showed hat there was a uniform distribution of glycogen in the lobules of cold-room bats nd a slight peripheral zonation of glycogen in the room-temperature animals. Never was there more glycogen in the central zone than in any other regions of he hepatic lobule.

Liver glycogen and blood sugar in adrenalectomized bats

Approximately 80% of the adrenalectomized bats survived the operation. It is observed (table 3) that the mean liver glycogen and blood sugar levels of the adrenalectomized bats which were returned to he cold-room immediately after the operation are significantly below those of the aparotomized control animals. Also, it appears that liver glycogen levels are lowered further when adrenalectomized animals are subjected to room temperatures, or the groups of adrenalectomized bats t room temperature for 20 hours before being returned to the cold-room, at room emperature for 12 hours after 92 hours n the cold-room, and at only room temperature for 15 hours had 28, 39 and 34 imes less liver glycogen, respectively, than did their laparotomized controls (table 3). t is also noted that the liver glycogen levels of the laparotomized animals are considerably lower if the animals were at room temperatures for a period immediately before they were killed. In addition to the fact that all differences in liver glycogen between adrenalectomized and laparotomized bats were significant ($p < 0.01$), the blood sugar levels of all of the adrenalectomized animals in this study were significantly ($p < 0.01$) below those of the laparotomized control animals.

The estimated amount of liver glycogen in the histochemical preparations of these experiments corresponded with the quantitative results in every case. In most of the adrenalectomized bats, glycogen was concentrated in cells only at the periphery of the hepatic lobule (fig. 9). All but one of the adrenalectomized animals, placed for a time at room temperature, had only minute traces of glycogen in a few scattered hepatic cells. Laparotomized animals, however, showed a paucity of glycogen only in those cells in the immediate vicinity of the central vein (fig. 10). In no case was more glycogen distributed in the central zone than the peripheral zone of the hepatic lobule.

DISCUSSION

Studies in normal bats

The increases and decreases in the liver glycogen levels obtained in this study only indicate physiological changes that take place in this hibernating animal during the various periods of its yearly cycle and do not offer an explanation toward the larger overall problem concerned with the causes and maintainence of hibernation. Certain speculations can be made, however, as to the origin and fate of these carbohydrate stores.

The observation that liver glycogen was at its highest level in bats of the first month of hibernation compares favorably with the results of investigators who found an accumulation of glycogen in other hibernators at the beginning of hibernation (Aeby, 1875; Luchsinger, 1875; Dubois, 1896). This peak is acquired relatively abruptly since liver glycogen in active summer animals was shown to decrease gradually to the very low levels of animals from the month prior to hibernation. In

conjunction with this, the observed increases in fat deposition and body weight in this study and other works on active bats of the month prior to hibernation (Beer and Richards, '56; Krutzsch and Sulkin, '58; Rémillard, '58) suggest a possible "filing-away" of carbohydrates in the form of fats.

If this speculation holds true, then the accumulation of liver glycogen in the first month of hibernation might be due to the last few feedings before hibernation or to glyconeogenesis from the large fatty deposits.

The general progressive decrease in the liver glycogen levels of bats observed from the first to the last month of hibernation compares favorably with previous studies on bats by Rulot ('02) in *Vespertilio murinus auct*, Reach ('10) and, Dodgen and Blood ('56) in *Myotis lucifugus lucifugus* and *Myotis grisescens*. Although the relative decrease in liver glycogen in this study is appreciable (68%) and contrasts with generalizations that liver glycogen levels vary only little in fasting hibernators during hibernation (Kayser, '50; Suomalainan, '56), the quantity of glycogen (2.94 gm %) utilized does not seem outstanding for this length of time. Since this bat is known to arouse from hibernation but remain fasting at various times during its hiberating period, there is a strong possibility that the glycogen decrease from November to May is not due to a slow utilization of the original glycogen stores, but is the result of a more rapid utilization which is being partially replenished by glycogen from non-carbohydrate sources. Although this phase of the study presents no evidence for or against the possibility that hibernating bats are capable of glyconeogenesis, the discussion concerning bats subjected to temperature changes and adrenalectomy deals further with this subject.

The significant decrease in liver glycogen levels of *Myotis lucifugus lucifugus* from the last month of hibernation to the first month of the active period (table 1) supports the conclusion that liver glycogen of hibernating animals decreases greatly at the moment of spring awakening (Kayser, '50). This comparison should be viewed with some reservation since liver glycogen in the present investigation wa not determined in bats as they arouse from hibernation, but was determined fc fasted bats which had probably fed afte waking from hibernation. This decrease i liver glycogen, however, does appear to b intimately associated with the increase activity and subsequent greater expendi ture of energy found in the awakened ba Blood sugar values obtained for the hi bernating and awakened animals of th months of May and June, respectively fail to throw any light on the mobiliza tion and utilization of carbohydrates dur ing the awakening process, since the ether killing method awakened the hibernatin bats and possibly increased their bloo sugar levels at the expense of liver glycc gen which was lowered after ether ac ministration (see Salter, '52).

Hibernating bats subjected to experimentally altered environmental conditions

In addition to the significant decrease in liver glycogen, arousal from hibernation and, increased activity observed in thi study in bats removed from the cold-room and maintained at room temperature fo 36 hours, Hock ('49) has measured a marked increase in the metabolic rate o *Myotis lucifugus lucifugus* over the same temperature range. Since there is genera agreement that increased muscular exer cise and metabolic rate are accompanied by an increased utilization of carbohydrate in mammals (Edwards et al., '34; Gem mill, '42), it is believed that the low liver glycogen levels of bats killed at room tem perature are the result of increased carbo hydrate utilization induced by an increase in the environmental temperature. In view of this, the restoration of liver glycogen to normal levels in bats returned to the cold room for 72 hours after 36 hours at room temperature indicates that these fasting animals are forming liver glycogen from non-carbohydrate sources, i.e., glyconeogenesis. The actual amounts of carbohydrate utilized and restored cannot be determined, since the lack of true blood sugars for the hibernating bats does not permit a comparison with those of active bats at room temperature. The other major factor limiting the validity of the

ata in this phase of the study is the inbility to use the same animal for liver lycogen determinations at both temperaure conditions.

Adrenalectomized hibernating bats

Since liver glycogen and blood sugar levls were considerably lower after adrenalctomy than after laparotomy, it appears hat the adrenal glands are necessary for naintaining carbohydrate levels in this hiernator. These results are in agreement vith those obtained in fasted adrenalectonized non-hibernators (see Soskin and evine, '52; Turner, '53). The bat, also, cts like non-hibernators (Britton and Silette, '34) in that measures which ordiarily stimulated hyperglycemic reactions n normal bats failed to do so in adrenalecomized bats. This was evidenced by the act that the ether-killing method did not aise appreciably the blood sugar levels f the adrenalectomized animals.

The data further indicate that adrenalecomized and laparotomized bats are simiar to normal bats in that liver glycogen evels were lower in animals subjected to oom temperature until killed than in nimals at cold-room temperatures. An xception to this similarity is found in the omparison of laparotomized and adrenalctomized bats that were returned to the old-room after 20 hours at room temperture. In that case the liver glycogen levls of the laparotomized animals appeared o be restored to the level represented by ats maintained only in the cold-room whereas the levels of the adrenalectomized nimals was more representative of anials killed after room temperatures. This lack of restoration of liver glycogen evels in the adrenalectomized bats suggests that bats are like non-hibernators in hat they are incapable of glyconeogenesis fter adrenalectomy (Long, '42).

The comparison of liver glycogen and lood sugar levels between all adrenalectonized bats subjected to room temperatures nd those maintained only in the cold-room urther indicates that this adrenalectonized hibernator is able to survive for a onger period of time at a low environmental temperature than at higher temeratures. Although this conclusion is in greement with work on other hibernators (Britton, '30), it contrasts sharply with findings in fasted adrenalectomized nonhibernators which show that the latter expends more energy when subjected to lower environmental temperatures (Hartman, Brownell and Crosby, '31). Thus, basic differences of thermoregulation between homoiotherms and hibernators (Benedict and Lee, '38) are maintained after adrenalectomy.

Histochemical determinations

The observed general progressive decrease in the amount of glycogen in the hepatic lobules of hibernating bats from the beginning of hibernation to the end of the active period undoubtedly is due to glycogenolysis. Since the decrease in glycogen is usually represented by a zonation with always more glycogen in the peripheral zone of the lobule than in the central zone, it appears that glycogenolysis occurs either more rapidly, earlier, or more extensively in the central zone than in the peripheral zone. This conclusion is supported by the fact that this same glycogen zonation pattern was found in bats subjected to conditions favorable for glycogenolysis, i.e., increased temperatures, adrenalectomy, and laparotomy. Also, Lyman and Leduc ('53) have found similar results in their work on the arousal of the golden hamster from hibernation. A possible explanation for this pattern of glycogen disappearance might be obtained from Deane's work ('44) on mice where she suggests that, "Glycogenolysis might begin in the central zone because of the relative anoxia of the region since, . . . glycogen is immediately broken down to sugar under anaerobic conditions."

Since the histochemical and biochemical results further indicate that the liver glycogen reserve is restored when room-temperature and laparotomized bats are returned to the cold-room, the lack of a greater concentration of glycogen in the central zone of the hepatic lobule suggests the possibility that glycogen is initially deposited in the peripheral zone with a secondary deposition in the central zone. This is similar to the glycogen deposition pattern described by Deane ('44) in the diurnal cycle of the mouse.

SUMMARY

1. Liver glycogen of female *Myotis lucifugus lucifugus* throughout the year, and under the experimental conditions of altered environmental temperatures and adrenalectomy was studied by biochemical and histochemical methods.

2. The liver glycogen levels of bats were highest in the first month of hibernation, showed a general gradual decrease during the course of hibernation, and were lowest in the last month of the active period. A significant decrease of liver glycogen was observed from the last month of hibernation to the first month of the awakened state. This decrease undoubtedly was associated with the greater activity and subsequent greater expenditure of energy found in bats at summer temperatures.

3. A striking similarity was found between the liver glycogen levels and body weights of the animals. An exception to this was a great increase in body weight, also indicated by an observed increase of fat deposition, during the month prior to hibernation which could suggest a possible "filing-away" of carbohydrates in the form of fats.

4. The validity of blood sugar determinations in hibernating bats killed by ether is discussed.

5. Evidence is given which suggests striking differences in the liver glycogen levels of bats from similar, but geographically different locations.

6. Liver glycogen levels were lowered markedly when hibernating bats were subjected to higher temperatures, adrenalectomy, and laparotomy. These glycogen and blood sugar levels were lowered further when adrenalectomized and laparotomized bats were placed in increased environmental temperatures. With the exception of the adrenalectomized bats, all animals returned to the cold-room after subjection to increased environmental temperatures had liver glycogen levels which were comparable to those of normal and laparotomized hibernating bats. The role of glyconeogenesis in this "restoration" of the liver glycogen level and the necessity of the adrenal glands for maintaining the liver glycogen and blood sugar levels of hibernating bats is discussed.

7. All of the histochemical preparations confirmed the biochemical determinations. In all cases the diminution of liver glycogen was represented throughout the hepatic lobule, but the loss was always greatest in the central zone of the lobule. There was never a greater concentration of glycogen in the central zone than in the peripheral zone of the hepatic lobule, regardless of whether or not the data suggested a deposition or loss of glycogen.

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PLATE 1

EXPLANATION OF FIGURES

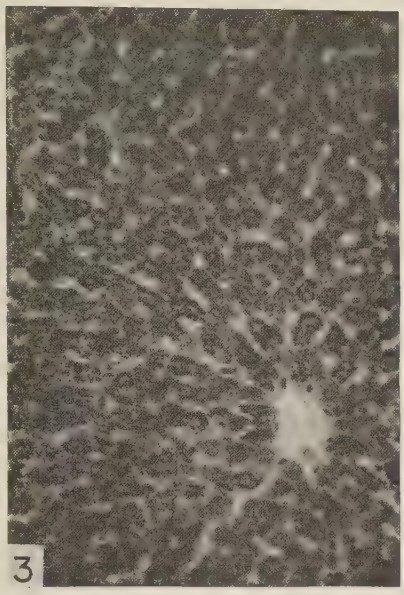
Photomicrographs of 6 μ sections fixed in Rossman's fluid and stained with periodic acid-Schiff's reagent for the demonstration of glycogen. A wratten B green filter was used to accentuate the fuchsin color and all pictures were printed to produce comparable color intensity.

3 Liver of a normal bat from the first month of hibernation. Glycogen is uniformly distributed throughout the lobule. Chemical determinations of glycogen: 3.20 gm%. $\times$ 260.

4 Liver of a normal bat from the first month of hibernation. Glycogen has been digested by saliva and no glycogen can be detected in the hepatic cells of this section. Chemical determination of glycogen: 7.09 gm%. $\times$ 260.

5 Liver of a normal bat from the fourth month of hibernation. The central zone of the hepatic lobule is in the middle of the field. Although no sharp glycogen zonation is apparent, slightly less glycogen is distinguished near the central vein than at the periphery of the lobule. Chemical determination of glycogen: 1.37 gm %. $\times$ 260.

6 Liver of a normal bat from the last month of hibernation. Glycogen is more heavily concentrated in the peripheral zone of the hepatic lobule than in the central zone Chemical determination of glycogen: 1.43 gm%. $\times$ 175.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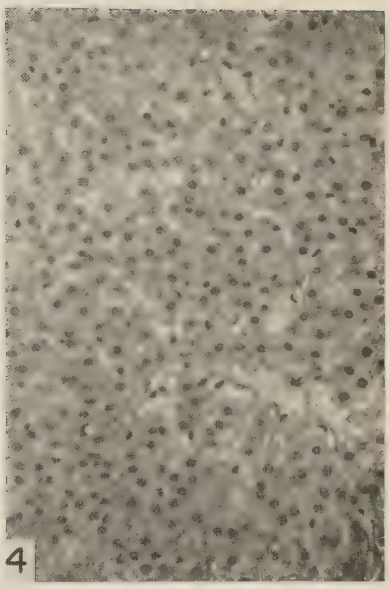
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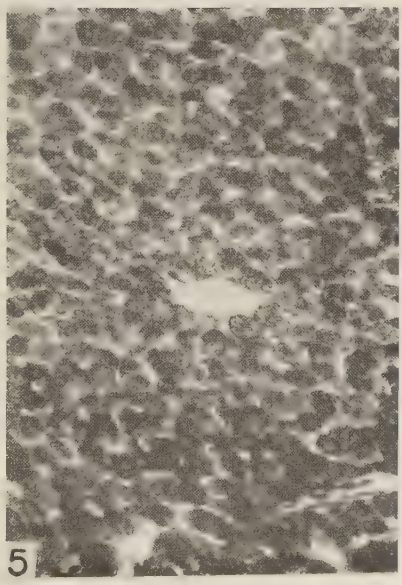
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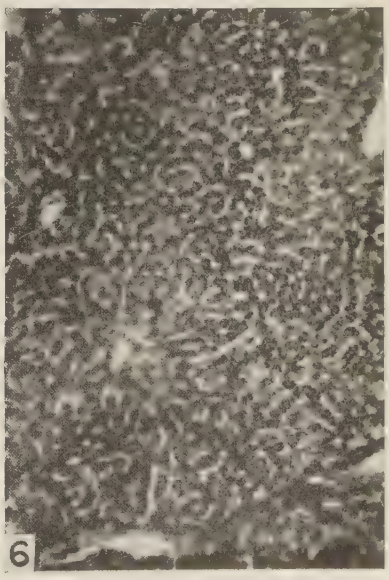
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PLATE 2

EXPLANATION OF FIGURES

7 Liver of a normal bat from the first month of the active period. The three most prominent blood vessels in the figure are central veins. A sharp zonation of glycogen is present with the greatest accumulation occurring in the peripheral zone of each of the hepatic lobules. Chemical determination of glycogen: 0.640 gm%. × 175.

8 Liver of a normal bat from the last month of the active period. The central vein of the hepatic lobule is above, the portal vein below. Glycogen is concentrated in a few scattered cells of the middle and peripheral zones of the lobule. Chemical determination of glycogen: 0.204 gm%. × 260.

9 Liver of an adrenalectomized bat. The concentration of glycogen is confined to cells in the immediate vicinity of the portal veins. Chemical determination of glycogen: 0.192 gm%. × 130.

10 Liver of a laparotomized control bat. Glycogen is rather uniformly distributed. Slightly less glycogen is found in the hepatic cells contiguous to each central vein, than in the hepatic cells of the middle and peripheral zones. Chemical determination of glycogen: 2.61 gm%. × 130.


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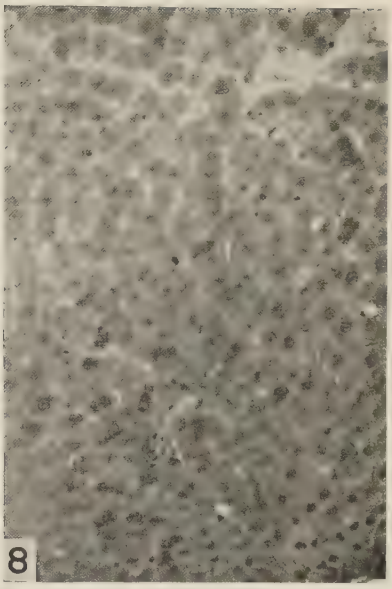
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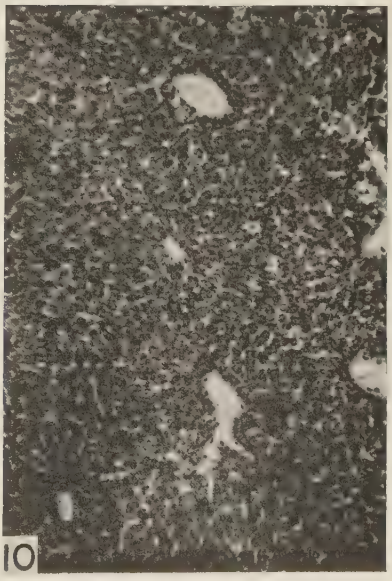
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he Effects of Elevated Temperatures on Yeast

NUTRIENT REQUIREMENTS FOR GROWTH AT LEVATED TEMPERATURES[1]

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The increase of nutrient requirements or growth at supraoptimum temperatures as been reported for a number of microrganisms (see general review by Precht, hristophersen and Hensel, '55, pp. 107, 92, 264). The most probable explanation f this phenomenon is the inactivation or hibition of the requisite enzymes at these igher incubation temperatures. Evidence or this hypothesis is available from the udy of Mass and Davis ('52) who have nown that the enzyme synthesizing pantoenic acid in a mutant of *E. coli*, which quired the vitamin only at temperatures ove 30°, was extremely thermolabile, mpared to the enzyme of the wild type, hich was not dependent on pantothenic id.

The present investigation is a study of e growth of yeast in various media at the timum temperature (30°) and at 40°C.

METHODS AND MATERIALS

Yeast cultures

The genetically marked diploid strain, 495, of *Saccharomyces cerevisiae* used in is investigation was obtained from Dr. K. Mortimer (Sherman, '58). A respirary-deficient (petite) variant, X495-S, was tained by first plating X495 and then lating a spontaneous occurring petite lony (Ephrussi, '53).

Preparation of growth media

In most cases, liquid growth media YED) contained 4% dextrose, 0.5% H_2PO_4 and Bacto-yeast Extract. Yeast-extract solutions were sterilized filtration, and dextrose and buffer solons were sterilized by autoclaving separtely for 15 minutes at 120°C. Solutions were stored at 4°C and mixed before using. This latter procedure was found to be necessary for obtaining reproducible results when growth at elevated temperatures was being studied. For example, no growth at 40°C could be obtained if a solution of 6% YED (6% yeast extract, 4% dextrose, 0.5% KH_2PO_4) had been stored at room temperature for a month.

Plating medium (PL), for the determination of viability, containing ½% yeast extract, ½% dextrose and 2% agar was sterilized by autoclaving. Preinoculation medium (PI) containing ½% yeast extract, 1% dextrose and 2% agar was similarly prepared.

Growth curves

In most experiments yeast was grown in 10 ml of medium placed in 50-ml flasks covered with beakers, and shaken in a Dubnoff metabolic shaking incubator (Precision Scientific). When this procedure was used, there was no evaporation even after several weeks of incubation. Unless stated otherwise the culture flasks were inoculated with 10^5 cells/ml of yeast which were previously grown for 24 hours on PI medium at 30°C. Under these conditions the inoculum consisted of about 98% single cells.

In the analysis of cell number, several criteria may be employed (see, e.g., Morris, '58). Turbidimetric methods, the most frequently employed technique, were not used in this study on account of the variability

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of transmission and turbidity of culture media, the difficulty of comparing viable and cell counts, and the change of cell size with different temperatures. Cell numbers were determined in these studies by (a) hemocytometer chamber count of cells plus buds, (b) hemocytometer count of clusters, (c) viable count by dilution plating method. Therefore, in the determination of a growth curve, aliquots were withdrawn from the culture flask at suitable intervals and analyzed by hemocytometer chamber count and by streaking a diluted volume containing about 100 viable cells on the surface of plates of PL medium. The streaked plates were incubated at 30°C for about 4 days for viable counting.

RESULTS

The growth of normal and petite strains in various amounts of yeast extract at 30°C, the optimum-growth-rate temperature, is shown in figure 1. Although the growth of the normal strain is relatively independent of the percentage of yeast extract, the petite strain shows a marked decrease in growth rate in 0.5% YED. Such decrease of growth rate is usually associated with the absence of a partial requirement in the medium (Kirsop, '55; Morris, '58).

When growth is studied at a higher temperature (40°) many quantitative and qualitative differences are observed. One interesting difference is that after one day incubation of the normal yeast at 40°C,

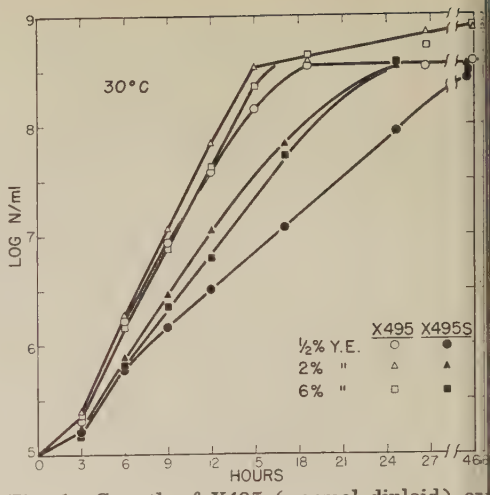

Fig. 1 Growth of X495 (normal diploid) an X495-S (petite diploid) at 30°C in 4% dextros 0.5% KH_2PO_4 and various amounts of yeast e tract. N refers to the number of cells plus buc

over 99% of the cells of the resultant po ulation are respiratory deficient. This ph nomenon will be elaborated in detail in later publication (Sherman, '59).

Another important difference is the gre dependence of growth of normal and peti yeast on the amount of yeast extract (fig 2, 3, 4). In 0.5% YED growth proceed only through a few divisions, with a dr in viability. Growth in 1% YED proceed similarly to that in 0.5% YED except th the initial death phase is only tempora and an increase of viable cells is observe after two days (fig. 3B). In higher pe

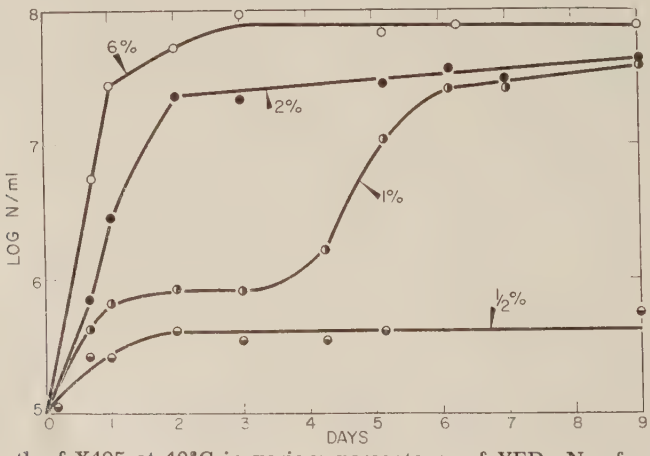

Fig. 2 Growth of X495 at 40°C in various percentages of YED. N refers to the number of clusters.

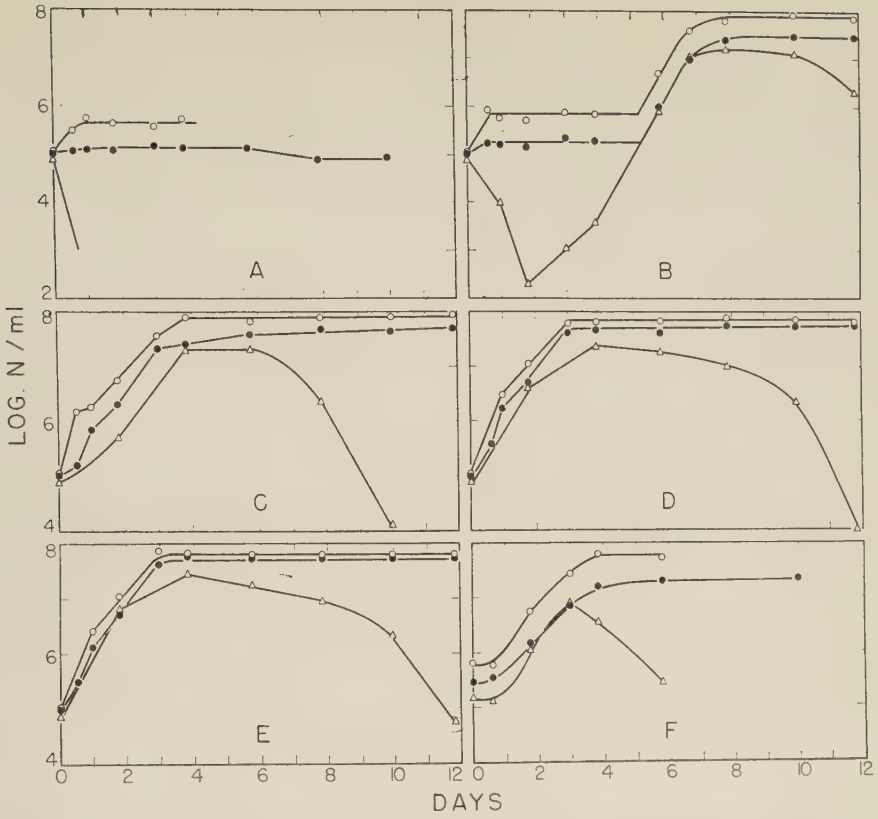

Fig. 3 Growth of X495-S at 40°C in YED of the following percentages: A, 0.5%; B, 1%; C, 2%; D, 6%; E, 8%. ○, cells plus buds; ●, clusters; △, viable count. F indicates growth in 0.5% YED in which the inoculum consisted of cells grown for 8 days in 1% YED at 40°C (curve B).

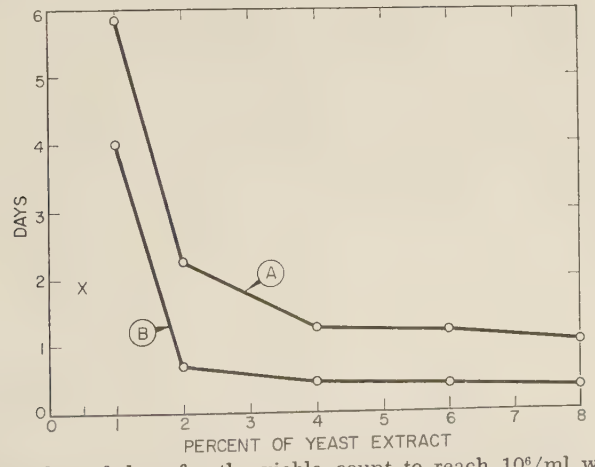

Fig. 4 The number of days for the viable count to reach 10^6/ml when yeast is grown in various amounts of YED at 40°C. A is X495-S (petite); B is X495 (normal). X refers to the acclimatized yeast (see fig. 3F).

centages of yeast extract, growth is relatively rapid. It should be noted that although the maximum titer of cells in stationary phase is independent of the percentage of yeast extract, the cells per cluster were found to decrease with increasing amounts of yeast extract. The death phase occurring after stationary phase was found to be rather pronounced at 40°C; no deaths were observed at 30°C even after one week of incubation.

Attempts to grow yeast in synthetic medium (Bacto Yeast Nitrogen Base, 1% dextrose) at 40°C were unsuccessful, and the resulting growth curves were similar to those of 0.5% YED.

If, after two weeks incubation at 40°C the 0.5% YED culture flask is transferred to 30°C, the growth proceeds rapidly to a titer comparable to that of figure 1. This indicates that the elevated temperature does not affect the growth medium and that the inhibition of growth is highly reversible. This is illustrated schematically in the top of figure 5. Also if cells grown

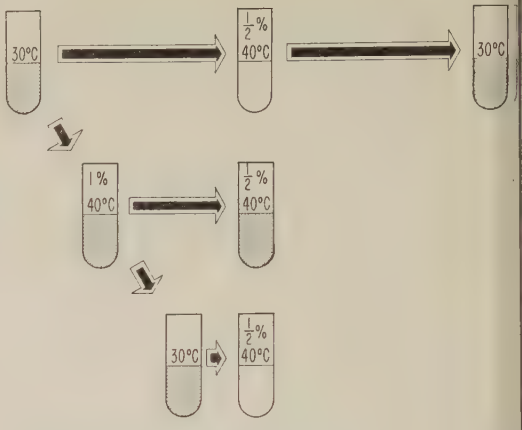

Fig. 5 A schematic representation of various experiments (see text). Stippling indicates growth.

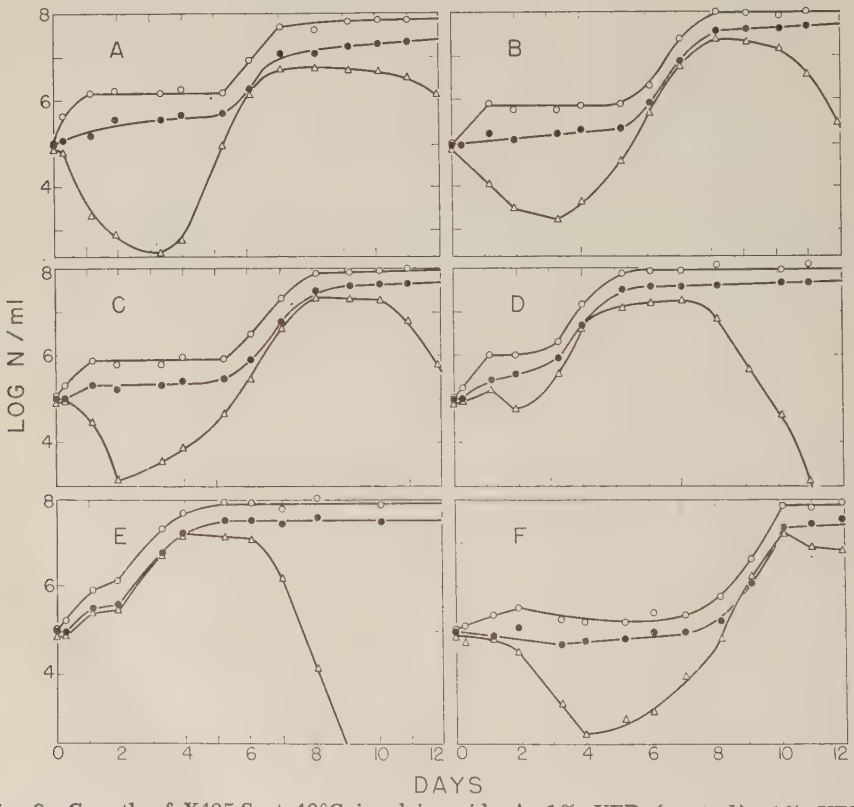

Fig. 6 Growth of X495-S at 40°C in oleic acid. A, 1% YED (control); 1% YED plus 1% ethonal with various amounts of oleic acid: B, 0; C, 0.1; D, 0.1; E, 1.0; F, 2.0 μmole/ml. ○, cells plus buds; ●, clusters; △, viable count.

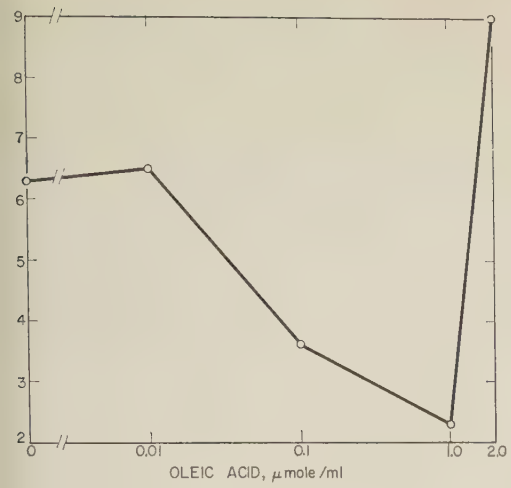

Fig. 7 The number of days for the viable count o reach 10^6/ml when X495-S is grown in 1% ED at 40°C in various amounts of oleic acid.

t elevated temperatures were used for the noculum, growth could be initiated in .5% YED. This is illustrated in figure F, in which the inoculant was yeast which ad been grown for 8 days at 40°C in % YED (fig. 3B). Furthermore, this lat- er effect can be lost if the elevated- emperature-grown cells are transferred nd grown at 30°C before inoculation in .5% YED. This is schematically repre- ented in figure 5, illustrating the reversi- ility of this adaptation process.

There are undoubtedly many nutrilites vhich affect the growth characteristics in imited amounts of yeast extract at elevated emperatures. Although no extensive in- estigation of this problem was under- aken, it was noted that ethanol solutions f oleic acid can greatly influence the growth curve in 1% YED at 40°C (figs. , 7). As seen in figure 6E the initial death hase can be entirely eliminated by the ddition of 1.0 μmole/ml of oleic acid. Addition of oleic acid in 0.5% YED, how- ver, could not initiate growth.

DISCUSSION

It has previously been found that opti- num growth of yeast is achieved by altera- ion of the media with different incubation emperatures. Stier and Scalf ('49) noted n increased lipid requirement for an- erobic growth of *Saccharomyces cere- visiae* at elevated temperatures. Pine ('57) found that at 35°C the yeast *Histoplasma capsulatum* is much more sensitive to vita- man deletions, mainly thiamin, than at 25°C. The optimum-growth-rate tempera- ture of yeast has been reported to be in- fluenced by varying the concentration of ammonium chloride (Buchanan and Ful- mer, '30, p. 82) potassium chloride (Bach- rach, '56) and sugar (Scarr, '51).

In the study of White and Munns ('51) yeast was observed to grow exponentially in synthetic medium over the temperature range of 20°C to 43°C. However, in order to induce growth at 43°C, a larger inocu- lum was required. The marked differences between the above study and the investiga- tion reported herein is undoubtedly due to different strains of *Saccharomyces cere- visiae*.

In this study marked dependence on the amount of yeast extract in the medium (figs. 2–5) was noted for growth at 40°C. This dependence was mainly manifested by occurrence of an initial death phase in low concentrations of yeast extract, which was not observed at the optimum-growth- rate temperature of 30°C. The growth rate was also influenced by the concentration of yeast extract, so that the optimum con- centration at 40°C was much greater (in the neighborhood of 8% YED) than at 30°C (about 2% YED).

The observation that under certain con- ditions oleic acid can completely eliminate the initial death phase is probably related to the change of lipid chemistry that occurs in yeast at elevated temperatures (Chris- tophersen and Kaufmann, '56). The mechanism of action, however, still re- mains obscure. It is of interest to note that oleic acid and other lipids are required for anaerobic growth (Andreasen and Stier, '54, '56; Bloomfield and Bloch, '58). Nevertheless, the effect described in the present investigation cannot simply be ex- plained as a decrease in the amount of available oxygen, since there is little change in the solubility of oxygen between 30°C and 40°C (Rahn, '32, p. 80). It also should be remembered that the petite vari- ant can grow adequately without addition of oleic acid in low concentration of YED at 30°C (fig. 1).

The death phase at 40°C was noted even in the absence of yeast extract, although

little loss of viability was observed when yeast was incubated in buffer at this temperature in the absence of both yeast extract and dextrose (fig. 5 in Sherman, '59). This latter finding seems difficult to reconcile with the observations that increased sugar concentration provides a protective effect against lethal temperatures (Precht, Christophersen and Hensel, '55, p. 243; Schelhorn, '56). The investigations of Schelhorn and others, however, were usually performed at above-maximum-growth temperature, in contradistinction to the present experiment. Since autolysis is quite marked at around 40°C (Joslyn and Vosti, '55) it seems justified to assume that the buffer was enriched with yeast extract from the "leaking" cells, although none was furnished initially.

With the above facts in mind, this death phase is strikingly similar to cell death occurring by "unbalanced growth" (Barner and Cohen, '57; Strauss, '58; Ridgway and Douglas, '58), i.e., death occurring when certain biochemical mutants are incubated in a medium devoid of their specific growth requirements.

The increased ability of acclimatized yeast to perform biological functions at elevated temperatures has been previously reported by a number of workers (see review by Sherman, '58). In the study reported herein, adaption is indicated by the increase of viable cells in 1% YED after an initial death phase, and the acquired ability of cells to grow in 0.5% YED after being acclimatized in 1% YED at 40°C. To explain these results, two mechanisms can be considered: (a) genotypic adaptation, i.e., selection of resistant mutants, and (b) non-inheritable phenotypic adaptation, i.e., de novo alterations of existing cells. These results are more consistent with phenotypic adaptation because if any thermophilic mutants were originally present in the inoculum, growth would eventually have proceeded in 0.5% YED. Also, the high degree of reversibility would indicate that these mutants would have a marked disadvantage at lower temperatures. It should be remarked, however, that selection has not been conclusively disproven.

One may conclude that growth at elevated temperatures results in an increased nutrient requirement which may be eliminated by induced adaptation.

SUMMARY AND CONCLUSION

The growth of yeast at 30°C, the optimum-growth-rate temperature, is relatively independent of the percentage of yeast extract. However, at 40°C no growth was observed in 0.5% YED (0.5% yeast extract, 4% dextrose), unless the inoculum consisted of cells which were previously grown at 40°C. In 1% YED growth proceeded only after an initial death phase, which could be eliminated by the addition of 1 μmole/ml of oleic acid. In higher percentages of YED, growth is relatively rapid.

No death phase was observed at 40°C if the yeast were incubated in buffer that prevented proliferation. Thus it would appear that this cell death is similar to lethality occurring by "unbalanced" growth.

The conclusion based on these observations is that growth at elevated temperatures results in an increased nutrient requirement which may be eliminated by induced adaptation.

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he Effects of Elevated Temperatures on Yeast

. INDUCTION OF RESPIRATORY-DEFICIENT MUTANTS[1]

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espiratory-deficient yeast arises sponta-eously from normal cells and under most onditions constitutes approximately 1% f a growing population. Such mutants are asily detected by their different colony norphology, being smooth, white, and also maller when plated on solid nutrient me-ium in which glucose is a growth-limiting actor. These "vegetative petite" mutants ave been extensively studied by Ephrussi nd co-workers and by others (for recent eneral reviews see: Ephrussi, '53, '56; herman, '58), who have shown them to be ytoplasmic mutants probably arising from n irreversible loss or inactivation of self-eproducing cytoplasmic units which are ecessary for the synthesis of cytochrome xidase and other enzymes.

Proof of non-Mendelian inheritance of egetative petites was furnished by genetic nalysis of diploid progeny resulting from cross of petite and normal haploid trains. Such diploid zygotes produce phe-notypically normal cells, as did the cultures erived from the 4 spores of a single ascus. uccessive backcrosses of the spores with he parental petite also resulted in evidence avoring a non-Mendelian interpretation. Even more direct confirmation of the cyto-lasmically inherited characteristics of egetative petites was furnished by exam-nation of bud clones from isolated hetero-aryons, which were found to have hanges in respiratory phenotype not asso-iated with recombination of nuclear mark-rs (Wright and Lederberg, '57).

Recent work of Ephrussi, Margerie-Hot-inguer and Roman ('55) has shown that here are two classes of vegetative petites, eutral (as described above) and suppres-ive. This latter type differs from the neu-ral petite in that it elicits a petite pheno-type when crossed with a normal cell. Petite strains, however, can have a variety of degrees of "suppressiveness" ranging from 0 to nearly 100%, i.e., per cent of zygotes giving rise to mutant clones. A decrease in suppressiveness can occur spontaneously or can be experimentally induced.

The rate of mutation to the vegetative petites can be enhanced by a number of chemical and physical agents (Lindegren, Nagai and Nagai, '58; Sherman, '58). The most extensively studied are certain acridines, which are highly active and specific mutagens. Ephrussi and co-workers have shown that a yeast population grown for 48 hours in the presence of acriflavine is composed almost entirely of petite cells, although in the absence of proliferation no effect was observed. It was further shown by the use of microtechniques that the phenomenon is due directly to a mutagenic action and not to selection (Ephrussi and Hottinguer, '51). Furthermore, this high mutation rate is somewhat preserved after the cells have been removed from the acriflavine. By using euflavine, Marcovitch ('51) has shown that the mutation rate can be very close if not equal to unity, i.e., all buds formed in the presence of the dye are mutants.

The investigation reported herein deals primarily with the production of vegetative petites by growth at elevated temperatures and by heat shock. Preliminary results of these effects have been previously

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reported by Ycas ('56) and the author (Sherman, '56).

METHODS AND MATERIALS

Yeast cultures

The 5 different genetically marked strains of *Saccharomyces cerevisiae* used in this investigation were obtained from Dr. Robert K. Mortimer. These were two haploid cultures, AS3, and S1796A, and three diploid cultures, X320, X495, and X674 (for genotypes see: Sherman, '58).

When petite variants of a strain were desired, the culture was plated and a spontaneously occurring petite colony was isolated. Unless stated otherwise the petite strains were transferred 7 to 8 times on preinoculation medium, with 24 hours of growth between transfers in order to stabilize them in growth rate (Harris, '56). The designation of such a derived petite strain is the strain number with a suffix "S."

Growth media

The preparation of liquid growth medium (6% YED; 6% yeast extract, 4% dextrose, 0.5% KH_2PO_4), plating medium (PL; 0.5% yeast extract, 0.5% dextrose, 2% agar) and preinoculation medium (PI; 0.5% yeast extract, 1% dextrose, 2% agar), have been described by Sherman ('59). Bacto-potato-dextrose broth (PD), which is identical to Bacto-potato-dextrose less agar, was specially ordered from Difco Laboratories.

Growth curves

Growth curves were obtained by hemocytometer chamber count and dilution plating method as previously described by Sherman ('59). By this latter method there was no ambiguity in differentiating normal and petite colonies. In this investigation all mixed or "scalloped" colonies (Ephrussi and Hottinguer, '51) were scored as normal.

Single-cell analyses

The analyses of single cells were conducted by methods similar to those of Ephrussi and Hottinguer ('51), and Spiegelman, DeLorenzo, and Campbell ('51), and consisted in isolating the progeny of single yeast cells by microtechniques.

The procedure used in this study was as follows: A coverslip was coated with 6% YED and 2% agar and then placed in a moist chamber. Twenty-four-hour PI-grown cells were streaked on a corner of the nutrient agar slab, and single cells were isolated by use of a microneedle (for description of the micromanipulator and microneedle see Burns, '56). After budding had occurred the desired cell was moved to a convenient location, where it was removed by a method of Fowell ('55) which consists of marking the coverslip above the single cell with a drop of ink, removing the coverslip from the moist chamber, and cutting the agar portion immediately below the marking. This entire procedure was conducted in a temperature-regulated box at 40.5 ± 0.5°C.

The agar block which contained the single cell was then placed in a test tube containing 0.5 ml of 6% YED and 0.5 ml of water, and incubated at 30°C. If growth occurred after 5 days of incubation the tube was shaken and either a diluted aliquot or a loop of cell suspension was plated on PL medium.

Heat shock

The cultures used in the heat shock experiments were prepared by growth at 30°C in liquid PD medium for three days. Survival curves were obtained as follows: The suspension of yeast was diluted to about 10^6 cells/ml in 0.5% KH_2PO_4 at the desired inactivation temperature (54 ± 0.01°C) and immediately agitated. Aliquots were withdrawn as a function of time and plated on PL medium.

The plates inoculated with the heat-treated cells were incubated for 4 or 5 days at 30°C, after which time colony counts were made. Unlike the growth experiments, these showed a spectrum of colony sizes. For this reason there was difficulty in differentiating the normal and petite colonies, and the "tetrazolium overlay technique" was employed (Ogur, St. John, and Nagai, '57).

Genetic analyses

The mating of cells was performed by the mass-mating technique, i.e., by mixing a loopful of the two cultures on PI medium. Eight hours after the crosses were made the mass-mating mixtures consisted mainly

zygotes and the parental haploid cells. this time the suspension was plated on thetic media in which only the proto- hic zygotes were able to proliferate mper and Burkholder, '49).

Sporulation was induced by transferring -day cells grown in presporulation me- m (2% Difco nutrient agar, 1% yeast ract, 5% dextrose, 2% agar) to sporula- n medium (0.3% sodium acetate, 0.02 % raffirose, 2% agar) and incubating at 25°C for one week (Adams, '49). Four- spore asci were then isolated and dissected by microtechnique.

RESULTS

Production of petites by growth at elevated temperatures

Growth of normal yeast at elevated temperatures resulted in an increase in the

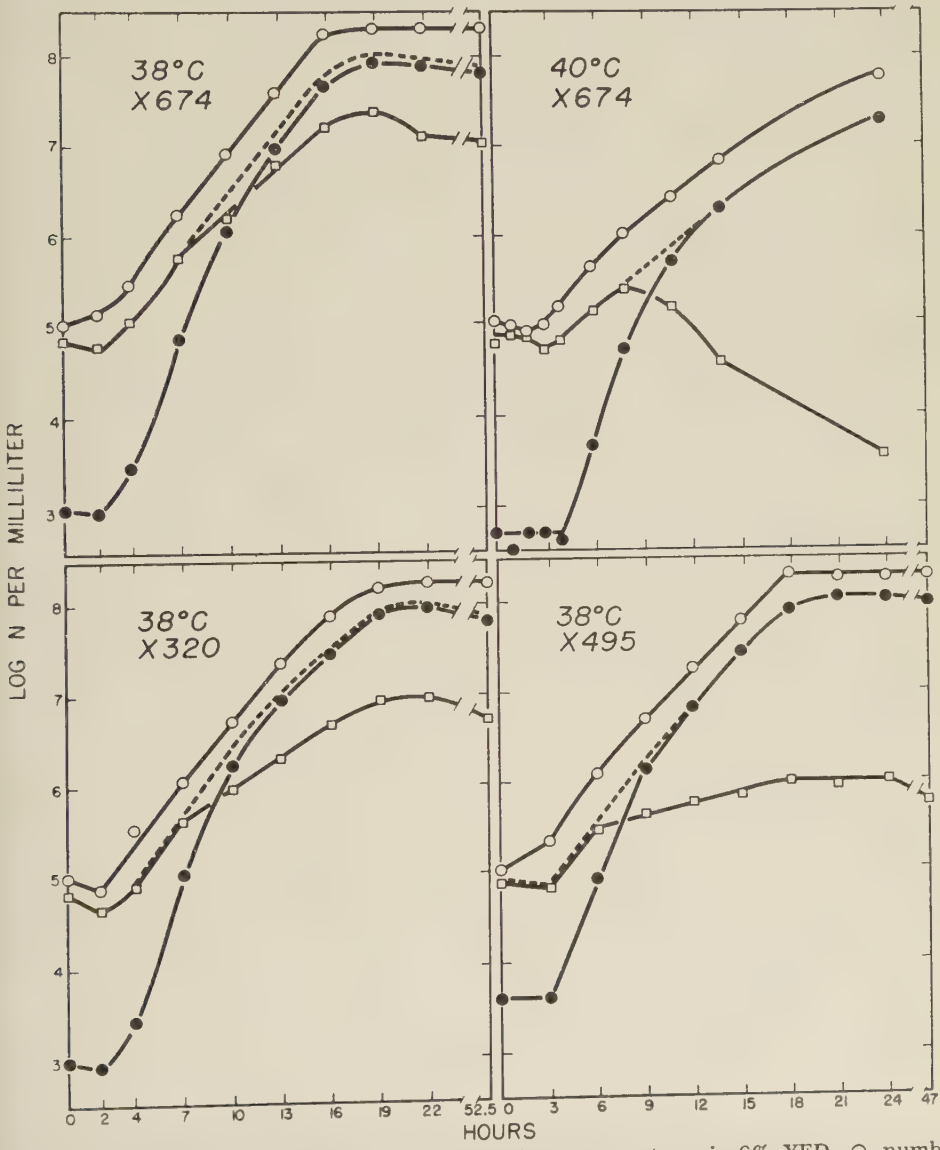

Fig. 1 Growth of various diploid strains at various temperatures in 6% YED. ○, number of cells plus buds; □, viable count of normals; ●, petites. Dashed line refers to the total viable count.

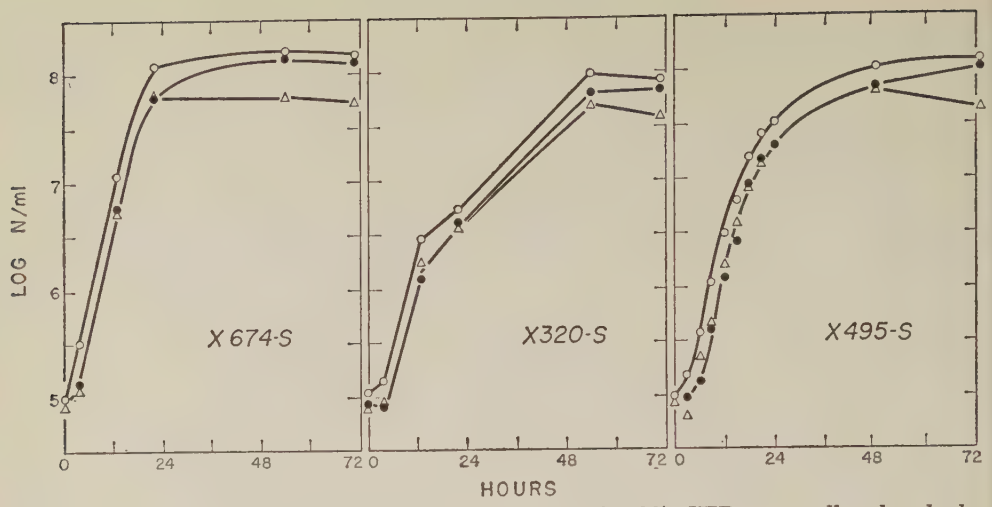

Fig. 2 Growth at 38°C of various petite strains in 6% YED. ○, cells plus buds; ●, clusters; △, viable.

fraction of petite variants, as ascertained by observing the colony forms. Besides the normal and petite colonies many "scalloped" or revertant colonies, containing mixtures of normal and petite cells, were also observed. This is also found in induction with acriflavine (Ephrussi and Hottinguer, '51).

Quantitative results of this phenomenon were studied by growing various strains of yeast at different temperatures in 6% YED (figs. 1 and 3). It can be seen that growth at these elevated temperatures results in an enormous shift in the population to predominantly petite cells. However, the corresponding growth curves of the petite strains (fig. 2) indicate that they have a longer generation time, about 1.5 times as long at 38°C as for a culture inoculated with primarily normal cells.

It is of interest to note the difference of petite production in the strain X495, which has a higher fraction of petites when grown at 30°C—10% in the steady state when grown in 6% YED, compared with 1% and 3% of strains X320 and X674 respectively. Also, it should be noted that temperature has less effect in the production of petites in the haploid strains.

Single-cell analyses

Although the shapes of the growth curves and the extreme efficiency of elevated temperatures in increasing the level of petite seem to be indicative of induction and n selection, this evidence is not conclusiv Direct proof of induction and its underlyin mechanism is furnished by the results the single cell analysis.

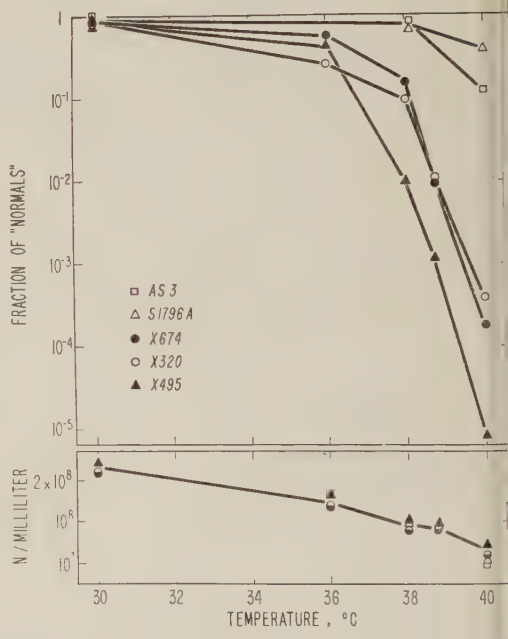

Fig. 3 (Above) Fraction of normals after 2 hours of growth with various diploid (X674, X32 X495) and haploid (AS3, S1796A) strains as function of temperature. (Below) Total viabl count after the same period of growth.

TABLE 1

ults of single-cell analyses at 40.5°C (see text)

Generations								
0	1	2	3	4	5	6	7	8
Daughter series								
+	+	+	−	−	△			
+	+	△	+	−	−			
+	+	△	+	△	−			
△	△	−	−	−	−			
+	+	+	−	△	−	△		
+	+	+	△	−	−			
+	+	△	△	−	−	−		
+	+	−	−	−				
Sister series								
+	+	+	+	+	+	+		
△	+	△	△	△				
△	+	+	+	+	+	+		
△	+	+	+	+	+			
△	+	+	+	+	+	+	+	+
△	+	+	+	+	+	+	△	+
△	+	+	+	+	+	△	+	+
△	+	+	△					
△	+	+	+	+	△	−	+	−
△	+	+	−	△	+	−	−	

+ = normal; − = petite; △ = inviable.

In this investigation two lineages at .5°C were considered:

(a) The *daughter series,* in which a ast cell was kept at the elevated tem- rature until the first bud could be re- oved. At this time the mother cell was ced at 30°C and the bud retained at .5°C until it budded, and the process s then repeated.

(b) The *sister series,* in which a yeast l was retained at the elevated tempera- re and its first, second, etc. buds removed incubation at 30°C. After the last bud s removed, and the sister series termin- d, the mother cell was also returned to °C.

At no time was there any confusion in e differentiation between a mother and daughter cell, mainly because at this nperature the mother cell always budded ior to its daughter, as previously re- rted by Burns ('56) for 38°C.

The results of these experiments are ven in table 1 and schematically illus- ted in figure 4. The daughter series early proves that the growth at elevated nperatures actually induces the muta- n and that it is *not* merely the ability petites to grow at a faster rate, i.e., lection. This is consistent with the results of the mass-culture technique. The data obtained indicate that the probability is very low for the original mother cell to produce petite buds. However, the first-generation cells produce about one-half petite buds. Whether or not the original normal cells can be induced to the petite phenotype could not be determined because of the high degree of inviability when they are transferred to 30°C.

Because of the method of the above study, a cell was considered normal when it gave rise to a clone containing as little as 0.1% normals. In order to determine the degree of heterogeneity, the resulting progeny of the single cells were plated out after the culture had grown to stationary phase (about 25 generations). The results of the first experiment of each series are shown in table 2 and seem to indicate that a high mutation rate was preserved over many cell generations. It should be remarked that with the method of cultivation of the single cells by a standing culture the percentage of petites is higher than by a shaking culture (aerated), being 10% and 1% respectively in a control study. It should also be remembered that under this condition of considerable growth, selection plays an important role, and if any normal cells were present, the culture would tend to approach a steady-state value of 10% petites. Therefore, if sampling were performed earlier, before many generations could have occurred, the fraction of petite variants was greatly enhanced.

In this investigation some interesting side observations indicated that elevated temperatures could induce many abnormal cell types. When log-phase cultures were

Fig. 4 Schematic representation of table 1.

TABLE 2

Per cent of petites resulting when single cells are taken from 40.5°C and allowed to proliferate at 30°C (see text)

	Generations					
	0	1	2	3	4	5
Daughter series	26.3	30.5	79.0	100	100	
Sister series	16.2	18.3	19.7	21.8	23.1	20.0
Control	10.5					

employed as inoculants, many filamentous cells were observed similar to the types induced by other agents (Morris, '58). Also, several dumbbell-shaped cells were observed which appeared identical to previously reported ultraviolet-induced types (Townsend and Sarachek, '53; Schauz, '58). These anomalous cell types were not observed with the liquid mass-culture technique.

Heat shock and induction of petites

An important question is whether the rate of mutation to the petite variant can be enhanced in nonproliferating cultures exposed to elevated temperatures. The result of such an experiment is shown in figure 5. In this experiment a 24-hour culture of 10^5 cells/ml was exposed to 40°C in the absence of yeast extract, with and without dextrose. Curves A and D of figure 5 show that in the absence of dextrose the viability drops only to about 50% in three days, with no significant change in the fraction of petites. However, in the presence of dextrose there is a marked drop in viability and a corresponding increase in the percentage of petites (fig. 5, B and C). This latter case may be similar to the previous experiments, in which low concentrations of yeast extract were employed in the growth medium (Sherman, '59), and although little growth took place there was a marked increase in the number of petites. It should also be remembered that at these temperatures autolysis takes place (Joslyn and Vosti, '55), and in the presence of dextrose there is probably a rapid turnover of yeast cells. One may therefore say that the marked drop in viability and the increase in the fraction of petites are occurring in a "growing" culture.

The conclusion that nonproliferating cultures exposed to elevated temperatures do not increase their yield of petite mutants was further exemplified when yeast was e posed to an above-maximum-growth te perature, 45°C. It was found that the was no increase in the fraction of petit even in the presence of yeast extract, though a drop in viability occurred. Th seems to indicate the independence petite production and cell death.

However, when the temperature of e posure is markedly increased, death p ceeds rapidly and a high percentage of su vivors of the heat inactivation develops in petite colonies.

In order to determine the relative sen tivity to lethal temperatures of normal a petite strains, survival curves of X320 a

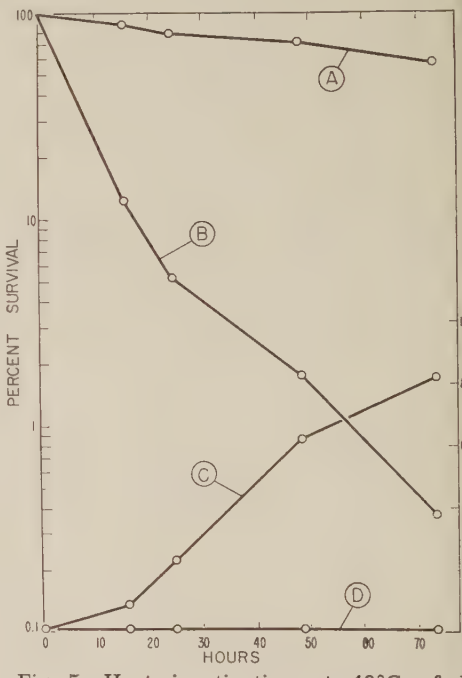

Fig. 5 Heat inactivation at 40°C of X6 (normal diploid): A, in 0.5% KH_2PO_4; D, wi the corresponding percentage of petites; B and in 0.5% KH_2PO_4 plus 4% dextrose.

320-S were determined at 54°C (Sher-
an, '56).

The petite strain was found to be ex-
emely temperature sensitive. However,
e petite strain X320-S was found to be
nstable with respect to its heat sensitivity,
r it became more resistant during storage
4°C. It differed from other petite strains
anomalous growth curves (at 30°C and
gher temperatures; see figure 2) and in
ving a lower maximum titer. Another
arked difference of this strain was that
eat-treated cells showed fewer survivors
hen plated on synthetic medium than on
east-extract medium. For the above rea-
ns the strain was abandoned and a sec-
nd petite strain was isolated, X320-S2.
he second petite strain was much more
sistant to heat shock than the original
etite isolate, but it should be pointed out
at in both cases the petite strains were
ways more sensitive than the related
ormal strains.

Figure 6 shows the results of inactivat-
ng three-day PD-grown cells at 54°C, and
lso the corresponding increase in the per-
entage of petites. Included for compar-
on is the survival curve of X320-S2-1, a
etite variant which was transferred once
PI medium. From the results of the
bove experiments it must be concluded

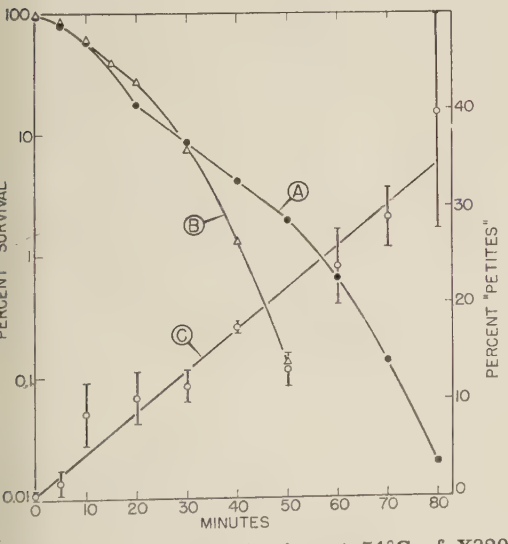

Fig. 6 A, heat inactivation at 54°C of X320
normal diploid) grown three days on liquid PD;
, with the corresponding percentages of petites;
is a similarly treated petite strain (X320-S2-1).

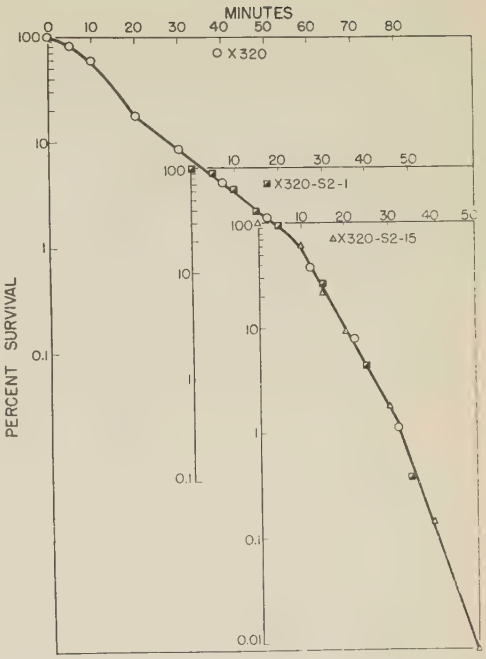

Fig. 7 Heat inactivation at 54°C of various normal and petite strains of X320. (Note that the axes have been shifted to allow coincidence of the curves.)

that the increase of petite mutants did not arise from selection, but was induced by the heat shock, since (a) petite strains are more sensitive to the heat treatment, and (b) there is an increase in the *absolute* number of petite colonies after a short exposure to heat. This latter point is illustrated by considering the results of the 10-minute exposure. The survivors of this treatment showed more than 10 times as great an absolute number of petite colonies as in the original inoculum.

Examination of figure 6 reveals the similarity of the petite survival curve to the tail of the normal survival curve. This similarity is illustrated in figure 7, in which the axis of the graph has been shifted to allow coincidence of the curves. Also included in figure 7 is a survival curve of X320-S2-15, the petite strain after it has been transferred 15 times on PI medium, allowing 24 hours between transfers. From this comparison one may say that the petite strains respond in much the same way as a population of normal cells that have survived a period of heat exposure.

TABLE 3

Suppressiveness of differently obtained petites. The per cent suppression is measured by the percentage of zygotes giving rise to petite clones minus the percentage of spontaneous petites in the normal strain, which is 6.8% for AS3 and 2.1% for S1796A

Origin of petite	Normal	X	Petite	% Petites	% Suppression
Spontaneous	AS3	X	S1796A-S1	32.5	25.7
	AS3	X	S1796A-S2	72.3	65.5
	S1796A	X	AS3-S1	58.4	56.3
	S1796A	X	AS3-S2	82.3	80.2
Acriflavine	AS3	X	S1796A-AC1	1.9	~ 0
	AS3	X	S1796A-AC2	94.0	87.2
	S1796A	X	AS3-AC1	2.2	~ 0
	S1796A	X	AS3-AC2	1.1	~ 0
Growth at 40°C	AS3	X	S1796A-H1	48.2	41.4
	AS3	X	S1796A-H2	61.3	56.5
	S1796A	X	AS3-H1	3.6	~ 0
	S1796A	X	AS3-H2	2.5	~ 0
Heat shock at 54°C	AS3	X	S1796A-T1	53.5	46.7
	S1796A	X	AS3-T1	35.8	33.7
	S1796A	X	AS3-T2	7.5	5.4

Genetic analyses

Several genetic tests were undertaken in order to determine the similarity of petites obtained by different methods. For this study several petite variants were isolated, from two haploid cultures of opposite mating type (AS3, S1796A), that had arisen by (a) spontaneous occurrence of petites (−S), (b) growth for 24 hours in 3% YED and 5 mg/1 acriflavine at 30°C (−AC), (c) growth for 24 hours in 6% YED at 40°C (see fig. 3) (−H), (d) heat shock at 54°C (−T).

Preliminary spectroscopic examinations of the differently obtained petite strains indicated that there were no obvious differences.

When these petite variants were crossed in all combinations (e.g., AS3-S1 × S1796A-H1, spontaneously occurring petites with petites obtained by growth at 40°C) the resulting zygotes produced only petite cells. Since it has been previously shown that spontaneous and acriflavine-induced petites are usually vegetative mutants, the conclusion is that these differently obtained petites were not supplemented by each other and segregational petites were not induced.

The degree of suppressiveness (Ephrussi, Margerie-Hottinguer, and Roman, '55) was obtained by crossing the various petites with the normal strain of opposite mating type and measuring the percentage of zygotes giving rise to petite clones (table 3). The data are insufficient to determine whether or not one method of induction produces a significant difference in the amount of suppressive petites.

The cross S1796A × AS3-H1 (normal × 40°C-induced petite) was sporulated and several 4-spore asci were dissected; 2:2 segregation of the histidine and tryptophane markers was observed, and all spores gave rise to normal clones, as would be expected for crosses involving vegetative petites.

From the results of these genetic analyses one may conclude that the petites obtained by heat shock and growth at elevated temperatures are very similar, if not identical, to the vegetative petites occurring spontaneously or induced by acriflavine. All methods of induction produce some suppressive petites.

DISCUSSION

Population analysis

In a population of cells the normal yeast cells are constantly mutating to the petite variant. The normal cells, however, enjoy a selective advantage, which, if large enough compared to the mutation rate, will result in a steady-state condition of a con-

nt fraction of petites. This is what oc-
rs normally at 30°C. However, if the
utation rate is sufficiently large, the
tites will eventually outgrow the normal
lls, as illustrated by growth at 38°C (fig.
. It is also possible that an absolute de-
ease in number of normal cells can re-
lt, as found with incubation at 40°C
g. 1). Therefore the resulting fraction
petites occurring by growth after a fixed
ne as a function of temperature, as de-
ieated in figure 3, involves all the above
ree situations.

One may ask if the decrease of normal
lls found at 40°C is due to the lethal
fect of the temperature, or to mutation
the petite variant. This is satisfactorily
nswered by examining the results of the
ngle-cell analyses (table 1), which indi-
ite that the original normal cells are pri-
arily lost by lethality and not by muta-
on.

Many similarities exist between the in-
uction of petites by euflavine (Ephrussi
nd Hottinguer, '51) and by growth at
mperature in the neighborhood of 40°C.
n both cases no increase of petites occurs
nless the cells are proliferating. Further-
ore, there is a qualitative resemblance in
hat there is a greater probability that a
ud will mutate than that the mother cell
ill. As indicated by the presence of "scal-
ped" or mixed colonies and from the re-
ilts of single-cell analyses, both methods
f induction produce an "unstable" state,
e., a state in which a high mutation rate
as preserved after the cells were removed
rom the inducing agents. A further pos-
ible parallel may be that the lethal and
nutagenic properties are independent, as
nown with euflavin by Marcovitch, ('53
,b) and by the experiment at 45°C in
hich lethality was observed with no in-
rease in the percentage of petites. Never-
heless, there are a few significant differ-
nces which may be of theoretical import-
nce. Before the details are discussed, a
ew remarks should be made about induc-
on in general.

In a growing culture of yeast the number
f normal cells, N_x, and petite cells, n_x, may
e given as a function of the number of
enerations, x, by the differential equa-
ons,

$$\frac{dN_x}{dx} = N_x \beta \ln 2, \qquad (1)$$

$$\frac{dn_x}{dx} = n_x \lambda \ln 2 + (1 - \beta) N_x \ln 2, \qquad (2)$$

where β is equal to the rate of increase of the log to the base 2 of the number of normal cells. In other words, instead of the normal cells doubling in number at each generation, a certain fraction mutates to the petite variant. The number of petite cells growing at a reduced rate λ can therefore be represented by equation 2. On the assumption that β and λ are constant, the solution for the number of petite cells as a function of the number of generations, x, is

$$n_x = n_o 2^{\lambda x} + N_o \frac{1 - \beta}{\beta - \lambda} (2^{\beta x} - 2^{\lambda x}). \qquad (3)$$

By taking the initial slope of the number of total cells as indicative of the generation time, one can plot the number of normal cells as a function of the number of generation. When this is done for X320 grown at 38°C (fig. 1) it is found that β has two values—initially 0.82, and after about 4

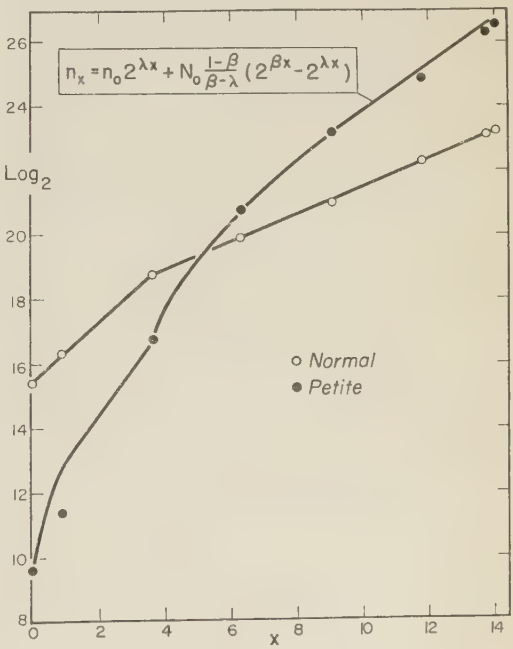

Fig. 8 Experimental points and the calculated curve of the number of petite cells as a function of the number of generations, x. Also shown is the experimental curve for the number of normal cells (see text).

generations 0.425 (fig. 8). If these two values are used for β, and λ is allowed to equal 0.64, the number of petite cells determined theoretically by equation 3 agrees with the observed number (fig. 8). One can therefore adequately describe the increase of petite cells in a growing culture at 38°C by assuming that the normal cells are constantly mutating to the petite variant and that the petite cells are growing at a reduced rate.

Mutation rates

In order to quantitate the induction of petites, Marcovitch ('51, '53a) has defined the "mutation rate," π, as the probability that a bud taken at random from a normal cell will be a mutant. The mutation rate of a steady-state culture with a constant fraction of petites, f_p, is given by Marcovitch ('53a) as

$$\pi = f_p(2 - 2^{\lambda}), \qquad (4)$$

where λ is the selection rate, defined as the ratio of the growth rate of petites to the growth rate of the normals. In the derivation of the above formula it is assumed that once a petite cell is formed, it will grow at a constant reduced rate. This assumption, however, may be only approximate, since Harris ('56) has found that in certain media the petites are characterized by a low initial growth rate, which increases after considerable growth. He also found a wide spectrum of generation times with different petite isolates of the same culture. With these facts in mind, one sees that the mutation rate calculated by the above formula is only approximate, and probably lower than the true value. With $\lambda = 0.71$ (Sherman, '59), the calculated values of the mutation rates of various diploid strains grown at 30°C in 6% YED are given in table 4.

If the mutation rate is higher, so that the petites are outgrowing the normal cells, as at 38°C, a different method of calculation must be employed. By the use of the relationship given by Marcovitch ('53a),

$$N_x = N_o(2 - \pi)^x,$$

and equation 1, one can demonstrate

$$\pi = 2 - 2^{\beta}, \qquad (5)$$

where β and its method of calculation are described above.

In table 4 the results of such calculations are shown for various strains grown at 38°C (see fig. 1). For example, these calculations show that in a steady-state condition about 90% of the buds produced by normal cells of strain X495 are petite mutants. Implicit in these calculations are the assumptions that lethality and cluster formation are independent of the cell types.

As noted in table 4 and figure 3, the relative order of mutability of the various yeast strains is different at different temperatures. For example, strain X495 has the highest mutation rate for all the temperatures studied except 36°C (fig. 3).

"Dilution" model

As can be clearly seen in table 4, the mutation rate at 38°C is initially very low and after approximately 4 generations reaches a higher steady-state level. This change of mutation rate, which is usually not found with the induction by certain acridines (Marcovitch, '51, '53a), can be best interpreted with various kinetic "dilution" models. Such models assume that there are initially a number of self-reproducing particles, which, under inducing conditions, cease to reproduce, or reproduce at a slower rate in relation to cell division. As a consequence, there is an increasing probability that a cell will possess either no particles or an insufficient number of particles as cell division proceeds and will therefore mutate. If there is a sufficient but low number of particles, the cell may produce both normal and mutant

TABLE 4

Mutation rates at 30°C and 38°C for various diploid strains

Temperature	Strain		
	X320	X674	X495
30°C	0.004	0.01	0.037
38°C initial (4 to 7 hours)	0.14	0.07	0.15
38°C steady state (7 to 18 hours)	0.65	0.39	0.87

ıds, and a resulting colony that is "scal-ped." Such models have been applied to xplain the elimination of kappa particles om *Paramecium* (Preer, '48) and the re-ersion of "long-term adapted" yeast (Spie-elman, DeLorenzo and Campbell, '51).

A general equation of this type, as modi-ed from Spiegelman, DeLorenzo and ampbell ('51) can be given as follows: it is assumed that a self-reproducing article has a probability p of being re-eived by a bud from the mother cell, and here is originally an average of θ_o par-cles per cell growing at a reduced rate , then the number N_x of normal cells t the X generation can be given by

$$N_x = N_o \sum_{j=\nu}^{\infty} \sum_{i=0}^{x} {}_xC_i \, f_j(\theta_{x,i}),$$

/here

= minimum number of particles necessary for a cell to be normal,

$${}_xC_i = \frac{x!}{i!(x-i)!},$$

$$\theta_{x,i} = \theta_o 2^{ax}(1-p)^x \left[\frac{p}{1-p}\right]^i,$$

nd $f_j(\theta_{x,i})$ is the probability of a cell's aving exactly j particles if the mean is $_{x,i}$. In order that the number of particles vill not increase in the mother cells, i.e., he growth rate of the particles is not reater than the effect of dilution, the fol-owing condition must be met:

$$(1-p) < 2^{-a}$$

As previously proposed by Spiegelman et al. ('51), the distribution function, $f_j(\theta_{x,i})$, can be assumed for the sake of simplicity to be equal to the Poisson distribution. The general formula can now be given as

$$N_x = N_o \sum_{j=\nu}^{\infty} \sum_{i=0}^{x} {}_xC_i \frac{[\theta_{x,i}]^j}{j!} \exp(-\theta_{x,i}). \quad (6)$$

The results of the mass-culture experiment of X320 grown at 38°C (figs. 1, 8) is used to illustrate the application of the above model to the induction of petites. However, this analysis is not presented in order to test the model, but rather—on the assumption that the model is correct—to determine the value of certain constants. The values of θ_o and a can be accurately determined by using equation 6 and assuming a certain ν (Appendix). The values of ν and p, however, do not appreciably affect the shape of the theoretical curves. Nevertheless, the best fit of equation 6 applied to the results of the X320 experiment leads to the values:

$$\nu = 1;\ p = \tfrac{1}{2};\ \theta_o = 3.36;\ a = 0.425.$$

This is shown in figure 9 along with the best-fit curves for $\nu = 1$, $p = \frac{1}{3}$ ($\theta_o = 3.36$, $a = 0.425$) and $\nu = 2$, $p = \frac{1}{2}$ ($\theta_o = 2.12$, $a = 0.713$). Correspondingly, on the assumption of $\nu = 1$, the values of θ_o for strains X674 and X495 (fig. 1) are 2.04 and 3.54 respectively.

As can be seen in figure 9, the theoretical curves tend to approach the limiting slope

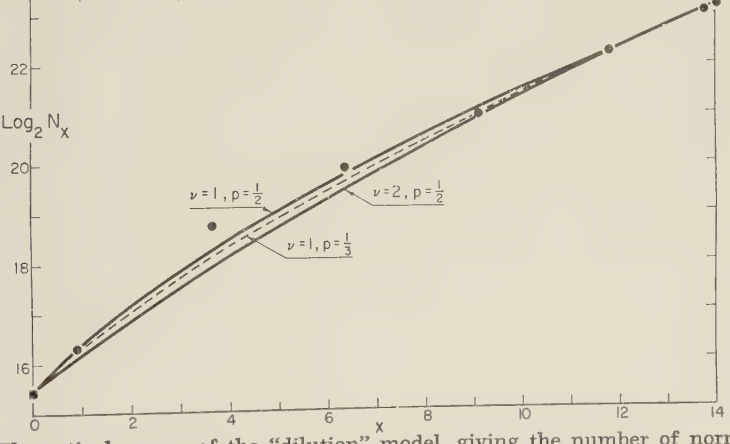

Fig. 9 Theoretical curves of the "dilution" model, giving the number of normal cells as a function of the number of generations, x; ν is the minimal number of particles required for the normal phenotype, and p is the probability that any given particle is transmitted to the daughter cell. Experimental points are for X320 at 38°C.

in a smooth continuous fashion. However, the experimental results are best described by two straight lines (figs. 1, 8). Whether or not this discrepancy disproves the model is still uncertain.

It should also be mentioned that these values of θ_o are not derived from cells at time equal to zero, but when $x = 0$, i.e., when the cells begin to proliferate. Since there is a slight initial death phase (about 80% of the cells surviving), it is possible that the original number of particles per cell was altered.

Although the results of the single-cell experiment are as yet insufficient for a complete analysis, a more sensitive evaluation of certain constants can be obtained from these data than from the mass-culture technique, (Spiegelman, DeLorenzo and Campbell, '51). Firstly, the approximate value of p may be calculated as follows: The average number of particles remaining in the x-generation buds of the daughter series can be given as

$$\theta_o 2^{\alpha x} p^x.$$

On the other hand, the average number of particles in x generation of the sister series is given by

$$\theta_o 2^{\alpha x} p(1-p)^{x-1}.$$

If the fraction of normal cells amongst x-generation buds of the daughter series is equal to the fraction of normal cells in the x′ generation of the sister series, then it can be shown

$$2^{\alpha x} p^x = 2^{\alpha x'} p(1-p)^{x'-1}.$$

Although the data as shown in table 1 are still of an approximate nature, one may take $x = 2$ for the fraction of normals to be on the order of ⅓, and x′ correspondingly may be in the neighborhood of 8 or 9. If the value of a is taken, as a first approximation, to be equal to the value calculated above for X320 at 38°C, then p can be shown to be equal to about ⅓. This value does not seem unreasonable, since at these temperatures it has been shown that the mother cell retains more than half of its cytoplasm during division (Burns, '56).

If one now assumes for simplicity that only one particle is necessary for a cell to be normal, the initial average number of particles can also be approximately calculated from the results of the single-cell analysis by using the previous equations. The results of such calculations lead to about 4 to 6 particles per cell.

Thus, on the basis of the above model, one is led to the conclusion that the number of self-reproducing particles per cell is low, around two to 6 per cell. This is in agreement with previous suggestions of various investigators (Ephrussi, '53; Pittman, '58). Slonimski ('53) has discussed other methods for the evaluation of the number of the self-reproducing particles.

It is quite possible that the above model is only an approximation of the real situation. Another model that could be considered is one in which the self-reproducing particles are attached to the cell wall, and the petite mutants develop from an area of the mother's cell wall which is devoid of such particles. Direct verification of the exact mechanism awaits a cytological investigation.

The induction of petites by growth at temperatures in the neighborhood of 38°C cannot be readily explained by the inactivation or inhibition of the cytochrome oxidase system, as this temperature lies far below the maximum for oxygen utilization (Tödt and Zimmermann, '57; and others). One must therefore conclude that the elevated temperatures act primarily by inhibiting enzyme formation. This is in correspondence with the results of various authors who have found that adaptive enzyme formation is more sensitive to elevated temperatures than enzyme activity present in adapted cells (Bernheim, '55). With this in mind, it would be of interest to investigate the adaptation to oxygen of anaerobically grown yeast at elevated temperatures, especially since this adaptation has been found to be blocked by euflavin (Slonimski, '53). Such investigations would help elucidate the site of action of elevated temperatures in the induction of petites, and specifically, if it is related to the meta-enzyme system, i.e., the catalytically active surface as proposed by Slonimski ('53) and by Ephrussi ('56) which may be responsible for the production of cytochrome oxidase.

Induction of petites by heat shock

The production of petites by heat shock at 54°C differs from that in the supraoptimum-sublethal range in that growth is un-

ecessary in the former. A possible interetation of this difference is that the divion of self-producing particles is merely hibited at the lower temperatures, while the higher temperatures these particles e inactivated.

The decrease of oxygen consumption at mperatures in the vicinity of 50°C is vidence that the cytochrome oxidase sysm is inhibited or inactivated in this range Sherman, '58). It has also been reported iat if yeast is heated at 80°C for 20 mintes, the cytochrome *a* and *b* bands disppear, while the cytochrome *c* band reiains (Ephrussi and Slonimski, '50). This of extreme interest if one remembers iat the petite variant is lacking in cytohrome *a* and *b*, but retains the thermoable cytochrome *c*.

Therefore, from a review of the literaire, it is apparent that the cytochrome xidase system is inactivated in the same emperature range in which petites are nduced in nonproliferating cultures.

The heat induction of petites is probbly a reflection of the thermolabile propty of yeast mitochondria. This is interestng since it has long been known that the nitochondria of plants and animals are ensitive to elevated temperatures. Heat hock around 40° to 50°C has been reorted to produce such changes as reducion of stainability, change of shape, and omplete fragmentation and disappearance f mitochondria in a number of different rganisms (see review by Sherman, '58).

Jefferson ('45) tentatively concluded hat the heat injury is due primarily to the reakup of mitochondria. It should be ointed out, however, that the petite muants may not have completely lost their nitochondria, as indicated by the existence f nonstainable refractile granules, and the resence of certain sedimentable enzymes, ut the mitochondria may merely be funcionally altered. It is also possible that the nitochondrial population is heterogeneous nd the mutation is the result of the loss f a fraction carrying cytochrome oxidase Ephrussi, Slonimski, and Yotsuyanagi, 55).

It seems appropriate to consider possible uantitative models for the analysis of the nduction of petites at lethal temperatures. A simple model can be given as follows: Initially there is an average of θ_o particles per normal cell, and these are inactivated by a first-order reaction, i.e., the same fraction of all particles that are surviving at any given time will be inactivated in the next time unit. Let it also be assumed that a cell has "mutated" when there is an insufficient number of particles per cell. If the inactivation of the particles is independent of cell death, and the sensitivities to heat of both normal and petite cells are of the same order of magnitude, then the fraction of petites will be very low for short exposures, increasing concavely upwards with time of exposure to inducing agent. Although the data (see fig. 6) are still capricious, it can be seen that the above analysis does not seem to apply to the induction of petites by lethal temperatures, but is best approximated by a linear relationship, i.e., $\theta_o = 1$. It would be presumptuous to perform an extensive analysis of the existing results, and a more complete interpretation awaits further data.

Other examples of temperature-induced non-Mendelian variants

Lederberg ('52) has proposed the generic term *plasmids* for "extra-chromosomal, intracellular hereditary factors, irrespective of their further identification as plasmagenes, viruses, self-reproductive organelles, endosymbionts, etc." As pointed out in his review, the disinfection of plasmids by differential heat treatment is quite prevalent. The mechanism may involve either *in vivo* heat inactivation or elimination by dilution if elevated temperatures inhibit or retard growth of the plasmids in respect to cell division. The heat induction of petites may be looked upon as a typical example of this phenomenon.

Similar situations are also found in *Paramecium* with kappa particles (Sonneborn, '46); in *Euglena* with chloroplasts (Pringsheim and Pringsheim, '52); in *Drosophila* for CO_2 sensitivity (l'Heritier, '58) and "sex ratio" (Magni, '54); in tomatoes for "rogue" formation (Lewis, '53); and others (Sherman, '58).

Heat inactivation of yeast

The heat-inactivation experiments of this investigation were directed primarily to the study of petite production. However,

a few remarks should be made on lethality *per se*.

The sensitivity of yeast to lethal temperatures is highly complex, and strongly dependent on the age of the culture, the type of medium in which the cells were incubated priod to the heat exposure, ploidy and strain (Sherman, '56, '58).

The quantitative results of the heat inactivation of the normal and petite strains, as illustrated in figure 7, suggest the following: If one assumes that a certain number of thermolabile sites have to be inactivated in order for death to occur, and that the petite has a relatively smaller number or an altered form of sites, then a petite culture's response may be similar to that of a population of normal cells that have survived a period of heat exposure. Also, since there is a difference between the responses of the strains X320-S2-1 and X320-S2-15, it is suggested that these thermolabile sites are lost or altered as vegetative growth of the newly formed petites proceeds.

From the above results it seems plausible to identify these thermolabile sites with the mitochondria. Although this seems highly speculative, it should be remembered that Jefferson ('45) has previously suggested this connection to explain his results with insect larvae, and to link the "enzyme" and "lipoid liberation" theories of heat injury. Claude ('41) has shown that incubation of extracts of dried yeast at 40°C for three hours results in a different type of sedimentable particle. There is also much evidence that the thermolabile property of ribonucleic acid is responsible for initial effects of elevated temperatures.

There is some disagreement on the exact nature of the mitochondria in yeast, but they are believed to be low in number and to undergo variation with the age of the culture. The above may help to explain the low multiplicity of the survival curves, and the large variations in sensitivity.

This highly speculative theory can be summed up as follows: if the mitochondria are partially destroyed by lethal temperatures, a mutation to the petite variant occurs; whereas, if the mitochondria are completely destroyed, lethality results.

SUMMARY AND CONCLUSIONS

1. When yeast is grown at supraoptimum temperatures, there is a great increase in the fraction of respiratory-deficient mutants (petites). However, in the absence of proliferation no increase was observed. By the analyses of the growth curves of the mass cultures and the single cell experiments, it was shown that this increase was not due to selection, but to induction by the elevated temperature. This high mutation rate continued for many generations, even after the cells were returned to the optimum temperature (30°C).

2. When the temperature of exposure was raised to above-maximum-growth temperature (45°C) no induction was observed. However, if the temperature was still further raised to extremely lethal temperatures (54°C), it was found that many petite colonies arose among the survivors. The conclusion that lethal temperatures induce petites is based on the observation that isolated petite strains were most sensitive to the lethal effect of elevated temperatures, and that an absolute increase in the number of petite colonies occurred after a short heat exposure.

3. Crosses of petite strains with each other and with normal strains were undertaken in order to determine the nature of the petite variants. It was concluded from the results of the genetic analyses that the petites obtained by heat shock and growth at elevated temperatures were similar, if not identical, to the vegetative petites occurring spontaneously or induced by acriflavine.

4. The results of the induction experiments can be interpreted as follows: There is initially in the normal cell a number of self-reproducing particles, which under supraoptimum temperatures cease to reproduce, or reproduce at a slower rate in relation to cell division. These particles can also be lost at lethal temperatures by heat inactivation. Therefore, a cell which possesses either none or an insufficient number of these particles mutates to the petite variant. On the assumption of the validity of a quantitative model (modified from Spiegelman et al.), it was found that the number of self-reproducing particles

cell is low, approximately two to 6 per
1.

5. It is suggested that the mitochondria
ıy an important role in the temperature
ıctivation of yeast.

APPENDIX

In order to illustrate the method of de-
rmining the constants of equation 6 with
e experimental results, a special case in
ıich ν is equal to unity is considered.
ıder this condition the equation reduces

$$N_x = N_o\left\{2^x - \sum_{i=0}^{x} {}_xC_i \exp(-\theta_{x,i})\right\},$$

which the symbols have the same mean-
g as in equation 6. When x is large, $\theta_{x,i}$
small and the above equation can be
ɔproximated by

$$\left\{2^x - \sum_{i=0}^{x} {}_xC_i\left[1 - \theta_o 2^{ax}(1-p)^x\left(\frac{p}{1-p}\right)^i\right]\right\}$$

emembering that we have,

$$\sum_{i=0}^{x} {}_xC_i = 2^x, \text{ and } \sum_{i=0}^{x} {}_xC_i(1-p)^{x-i}p^x = 1$$

ıe can simplify the above equation to

$$N_o\theta_o 2^{ax},$$

ıd a is equal to β of equation 1 when x
large.

By plotting the experimental results as
g to the base 2 of the number of normal
ells as a function of the number of gen-
rations, one finds that the limiting slope
equal to a. If a straight line is extended
om this limiting slope to $x = 0$, the
xtrapolated value is equal to $\log_2 N_o\theta_o$.
Vith this method θ_o and a can be accu-
ıtely determined.

Although slightly more complicated, sim-
ar methods can be employed for ν greater
ıan unity.

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ne Effect of Temperature on the Isolated Hearts of losely Related Hibernators and Non-hibernators[1]

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It is generally agreed that one characteristic of deep hibernation in mammals is a profound lowering of body temperature to a few degrees above the freezing point of water. The fact that the hibernator remains alive under these conditions shows that the vital processes must continue at very low temperatures and the evidence points to a general rule that the tissues of mammals which hibernate are peculiar in their ability to function at temperatures approaching 0°C (Tait, '22; Chatfield et al., 38). As early as 1881 Horvath recognized this specialization and suggested that all such mammals could be separated from the non-hibernators by their ability to tolerate hypothermia. If this is the case, it is of some interest to explore whether the ability to function at low temperatures constitutes a basic difference between the tissues of hibernators and mammals which do not hibernate.

Although low body temperatures in various hibernating mammals are well documented, the information on the tolerance to hypothermia of non-hibernating mammals is relatively scanty. It is limited almost entirely to the common laboratory mammals, of which only a few belong to the order Rodentia wherein the great majority of hibernators are found. The present study was undertaken to discover if the tolerance to extreme hypothermia was actually a physiological characteristic of tissues of hibernators alone, or whether it also existed in closely related mammals which were known not to hibernate. Furthermore, by choosing species of non-hibernators with various degrees of taxonomic affinity, it was hoped that some clues to the phylogenetic development of tolerance to hypothermia could be found. Finally, the difference in the tolerance of tissues to hypothermia among various species of hibernators was explored.

Mammals from the order Rodentia were chosen because hibernators and non-hibernators which are closely related phylogenetically were available for study. The isolated heart was used to test the tolerance to hypothermia since current research indicates that this tissue is particularly sensitive to cold.

Because our preliminary experiments indicated that the tissues from animals which hibernate are indeed more tolerant to cold than are the tissues of non-hibernators, a series of experiments was designed to exclude the influence of the host. Tissue cultures were made of the hearts of newborn hamsters and rats and, when spontaneous contraction occurred in these cultures, they were exposed to lower temperatures.

MATERIALS AND METHODS

The non-hibernating rodents used in this study were the grey squirrel (*Sciurus carolinensis*), the laboratory rat (*Rattus norvegicus*) Hisaw strain, the cotton rat (*Sigmodon hispidus*) and the mountain beaver (*Aplodontia rufa*). The hibernators were the golden hamster (*Mesocricetus auratus*), the 13-lined ground squirrel (*Citellus tridecemlineatus*), the chipmunk (*Tamias striatus*) and the woodchuck (*Marmota monax*). The phylogenetic relationships of these rodents are illustrated graphically in figure 1 following Simpson's

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[2] We are grateful to Mr. Howard Cramblett of Eureka, California, who gave us the rare mountain beavers.

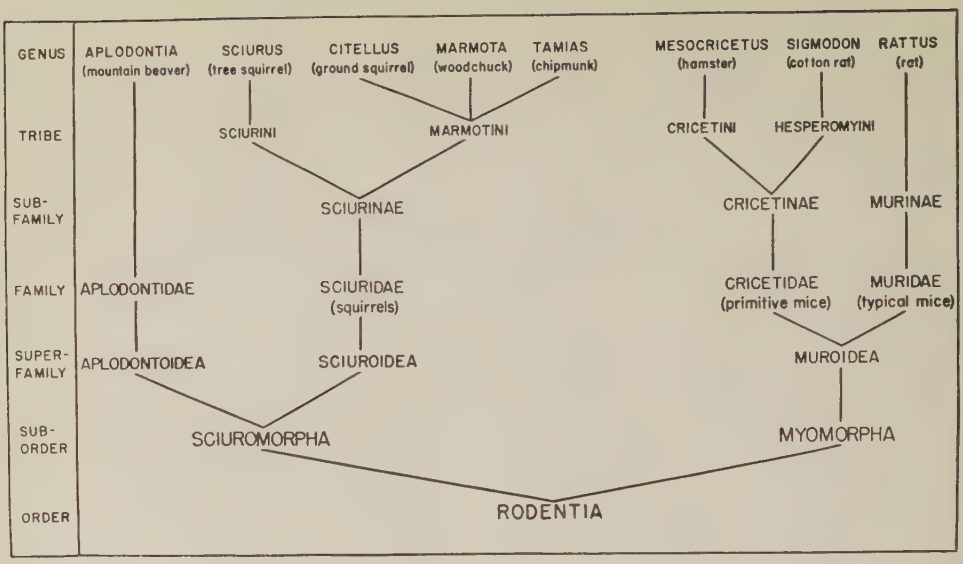

Fig. 1 Diagram showing the phylogenetic relationships of rodents used in this study.

classification ('45). The grey squirrels, chipmunks, and woodchucks were trapped locally, while the ground squirrels were trapped in Iowa and the mountain beavers were taken in northern California.[2] The other animals were obtained from dealers. The chipmunks were fed a commercial hen scratch feed, the mountain beavers were given fresh carrots, dandelion greens and Purina rabbit pellets, and the other animals were kept on Purina laboratory chow.

In order to provide a comparison with the isolated heart rates, individuals of each species except the mountain beavers were lightly anesthetized with ether and fitted with three electrodes, each taped to a leg. The electrocardiogram (EKG) was taken while the animal was in a steady state under light anesthesia, and the lowest rate for one minute was considered typical. After recovery, the lowest rate recorded for the undisturbed intact animal in a covered cage was recorded as the "normal" resting rate.

In preparing the isolated hearts of active animals, the best results were obtained by heparinizing (0.1 mg heparin/K) the etherized animal and applying artificial respiration. The hibernating hamsters were untreated before operation. The hearts were perfused through the aorta with Krebs-Henseleit solution (Krebs and Henseleit, '32), which had been previousl gassed with 95% oxygen and 5% carbo dioxide, and this atmosphere was main tained over the perfusion fluid (Albritton '46). Perfusion pressure was 106 cm c water. Ten to 20 minutes were usually re quired to remove the heart and start pe fusion.

Fine stainless steel electrode clips wer fastened to the heart so that the beat wa unimpeded, one at the base of the righ ventricle, one on the stump of the ven cava, and one on the stump of the in nominate artery. The EKG was recorded o a three-channel Grass ink-writing oscillo graph. The heart was placed in a sma temperature-controlled chamber, and th temperature of the heart, as measured a the tip of the perfusion cannula, and th chamber were recorded every 32 second by means of a Leeds and Northrup Speedo max recorder (Type G) calibrated to = 0.25°C. The preparation was heated o cooled at a rate of 0.2 to 0.5°C per minute After each change, the temperature wa stabilized for 5 minutes and the hear rate at the end of this time was used i plotting the data. Measurements of th duration of the P-wave, P-R interval, an QRS complex were made on representativ hearts of each species except the mountai beavers, but these could only be approx

ate at low temperatures because deflec-ons from the base line became progres-vely less sharp and additional deflections ere apt to appear.

Attempts to culture adult tissue were un-uccessful, but good cultures were obtained om the hearts of day-old rats and ham-ers using the coverslip technique. Ex-lants were fastened to the coverslips with nicken plasma, and the tissues were incu-ated at 37°C in Carrel flasks to which ere added 16 to 20 drops of culture me-ium. The medium consisted of 7.5 cm^3 agle's solution, 2.0 cm^3 horse serum, 0.5 m^3 chick embryo ultrafiltrate, and three rops pH indicator, and this was changed very three or 4 days. After 10 or nore days of incubation, areas in the cul-ures began to contract spontaneously and ontinued to do so for many days.

The rate of contraction at various tem-eratures was observed under a projection nicroscope in an apparatus similar to that lescribed by Smith et al. ('51). This ap-aratus consisted of a thick copper plate vhich surrounded the sides and part of the op and bottom of the culture flask. The wo ends of the copper plate were bent to xtend downward over the sides of the nicroscope stage and into two wide-nouthed thermos jugs filled with 95% alcohol. To lower the temperature in the culture flask, dry ice was added to the alcohol. A thermocouple resting 2–3 mm from the culture indicated the temperature by means of the Speedomax recorder. The changes in temperature were carried out as described for the isolated hearts.

RESULTS

Figures 2 and 3 indicate the relationship of temperature to the heart rate in 7 of the species tested. It is clear that the hearts of animals which hibernate (fig. 2) show activity at a lower temperature than do the non-hibernating animals (fig. 3), but that the temperature characteristics are different for each species. The responses of the hearts of each species are described below.

Ground squirrel

The resting heart rate of two intact ground squirrels varied between 192 and 234 beats per minute. Under anesthesia the rates varied from 330 to 372 beats per minute. The results from 5 isolated hearts were almost identical. The rate varied linearly with temperature to about 13°C, but below this the relationship became curvilinear (fig. 2). At −1°C, which was the limit of the recording device, the hearts beat with a complete, slow EKG and an

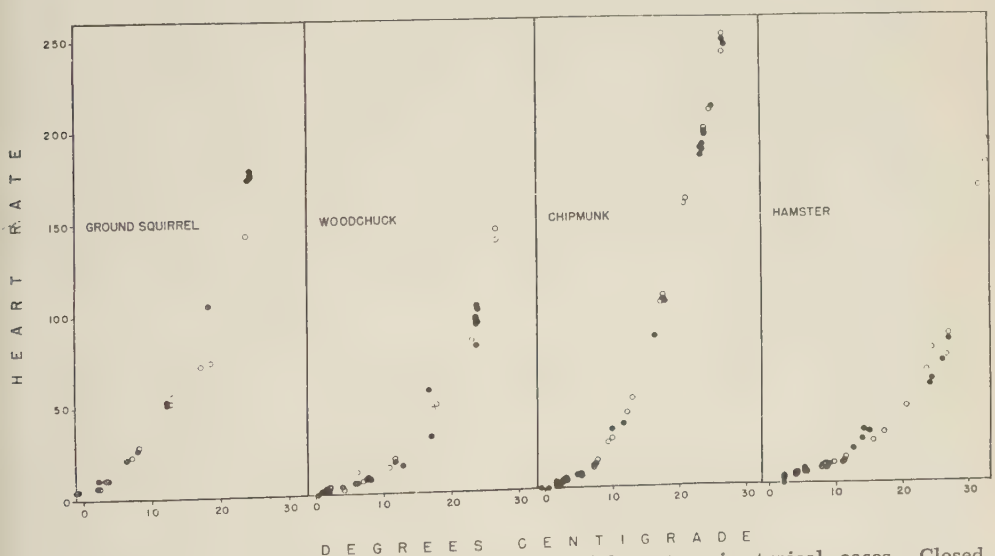

Fig. 2 Effect of temperature on heart rate of hibernators in typical cases. Closed circles = chilling, open circles = rewarming the heart. In the chipmunk, ventricular arrest occurred between 5° and 7°C and atrial rate only is plotted below this temperature.

even rate. The hearts functioned at low temperatures even if the rate of chilling was rapid, as in one case when the temperature was lowered from 28°C to 1°C in an hour.

Woodchuck

The resting heart rates of two intact woodchucks were both 180 beats a minute. This is almost twice as fast as the rates of woodchucks fitted with chronically implanted electrodes (Lyman, '58). This may be due to nervousness of the animals reported here. When the animals were under anesthesia, the heart rates varied from 242 to 265 beats a minute. Although it is not obvious in figure 2, the rates of all 4 of the isolated hearts varied almost linearly with temperature from 28° to approximately 15°C. Below 15° the line relating heart rate to temperature became curved. One heart was still beating at −0.5°C (fig. 2); two hearts stopped at 0.5°C, and one ceased at 4°C. In the latter case only the ventricular complex was recorded in some of the beats just before the heart stopped, but the EKG of the others was normal at low temperature.

Chipmunk

The resting heart rates of two chipmunks were 187 and 260 beats per minute, while the rate of both animals stabilized at 450 ± 2 beats per minute during anesthesia. The temperature-rate curves of the 4 isolated hearts all varied linearly with temperature from 30° to 12°C, but curved rather abruptly below this temperature (fig. 2). Between 5° and 7°C, the ventricles ceased to beat, but the atria were more resistant to cold and stopped between 1.7° and 3.3°C.

Hamster

The normal resting heart rates of two hamsters were 252 and 264 beats per minute. Under ether anesthesia the rates varied between 342 and 394 beats a minute. Difficulty was experienced in obtaining repeatable temperature-rate curves from the isolated hearts of hamsters, a peculiarity that has also been reported by Adolph ('51a). This appears to be due to an inherent delicacy of the heart. The relationship of rate to temperature in successful preparations was curvilinear ev at high temperatures, although the cur became more abrupt at low temperatu (fig. 2). In 8 representative experimen the last beats were recorded at temper tures between 0° and 4°C. Usually atr ventricular dissociation was followed ventricular arrest, with only the atria co tinuing to beat for the last two or thr degrees. In two hearts a normal sequen of depolarization was present until t heart stopped beating between 2.8° a 3.3°C. Occasionally at low temperature normal sequence of depolarization was r corded without a visible contraction the heart muscle. There was no differen in the response of hearts from golden a albino hamsters.

An extended study was made of t effect of cold on the isolated hearts fro animals which were in deep hibernatio These hearts were extremely sensitive manipulation, but did not differ from th hearts of non-hibernating hamsters in the ability to function at low temperature Eight hearts were colder than 13°C whe removed from the animals and were the chilled until they ceased to beat (betwee 1° and 4.5°C). Only three of these heart when warmed, responded with the ten perature-rate curve which had been esta lished for the hearts of non-hibernatin animals. Warming the isolated hearts fro 4 hibernating animals to 17°C or abo before chilling them improved their r sponse to some extent when they we warmed for the second time. The hear from two awake hamsters which had bee in hibernation recently responded like th hearts of control animals. The reason f the sensitivity to manipulation in th hearts of hibernating hamsters is n known.

Rat

The resting heart rates of two inta rats were 324–342 beats per minute. Whe the animal was anesthetized, the hea rates varied between 315 and 360 beats p minute. In all of the isolated hearts th rate varied almost linearly with temper ture (fig. 3). One heart showed an orga ized beat at 9.2°C, but in 6 others electric activity disappeared between 10.6° an 15°C. Usually the beat, though extreme

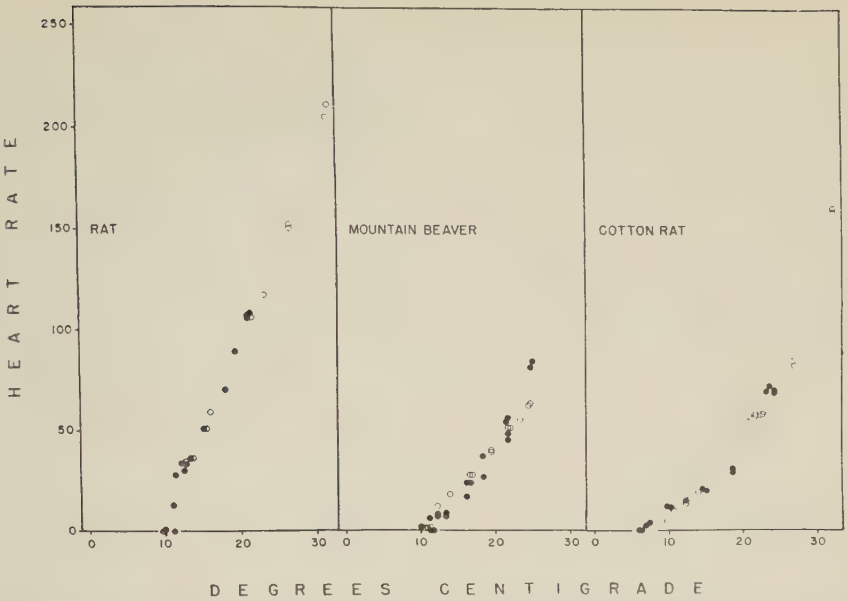

Fig. 3 Effect of temperature on heart rate of non-hibernators in typical cases. Symbols as in figure 2. Ventricular activity was not seen below 12°C in the cotton rat and atrial rate only is plotted below this temperature.

regular, had the normal sequence until ıe heart stopped, but in two hearts the ria continued to beat after the ventricles ad stopped, and in one the atria stopped rst.

Mountain beaver

At temperatures above 16°C, the ven-icles of the isolated hearts of two moun-ain beavers tended to fibrillate, but at ower temperatures an organized sequence f depolarization occurred. The plots of emperature against rate of both hearts ere almost identical, with the lowest tem-erature at which beats were recorded eing 10.6°C (fig. 3). The EKG had a ormal sequence until the hearts ceased eating.

Cotton rat

The normal resting heart rate of a single ntact cotton rat was 341 beats per minute. When the animal was anesthetized, the ate was 435 beats per minute. In the uni-orm results from 5 isolated hearts either he ventricles or the A-V node was ad-ersely affected by cold, for the ventricular omplex ceased between 18° and 12°C. While the hearts were beating normally, he relationship of temperature to rate was almost linear, but the line began to curve slightly when the atria alone were beating. Although the atrial beat was often the only activity to reappear when the heart was rewarmed, this was probably due to deterioration of the preparation, for hearts from cotton rats perfused at 23–25°C lasted only 2½ hours while hearts from Norway rats still functioned after more than 4 hours.

Grey squirrel

The heart rates of two grey squirrels under anesthesia varied from 300 to 408 beats per minute, while the resting rate was between 132 and 156 beats.

Of all the species tested only the hearts of the grey squirrels produced more than one type of result. Usually, if the heart was cooled at 0.3° or 0.4°C per minute, the relationship of heart rate to tempera-ture was linear and the heart stopped beat-ing between 13° and 16°C (fig. 4, left). Usually both atria and ventricles ceased at the same time, though sometimes the ventricles continued to beat at slightly lower temperatures.

However, in 6 of the 16 hearts studied, some activity was recorded below 7°C. Cooling at about one-half the usual rate

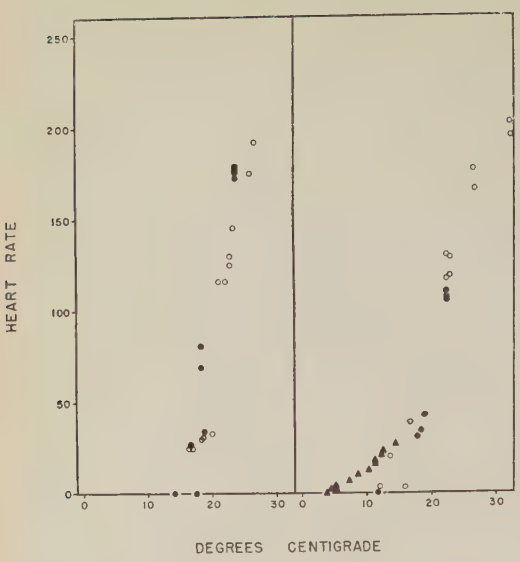

Fig. 4 Left: Typical effect of temperature on heart rate in grey squirrel. Symbols as in figures 2 and 3. Right: Effect sometimes seen in hearts of grey squirrels. Closed circles = chilling; open circles = rewarming; closed triangles = chilling a second time, ventricular rate.

often resulted in activity 5–11°C lower than could be elicited if the same heart were cooled rapidly (fig. 4, right), and this was accompanied by changes in the EKG. Between 13° and 16°C the QRS complex appeared before the P wave, the time sequence and shape of the complex indicating that the normal sinus rhythm had been replaced by a rhythm originating in the A-V node. At this time the P wave often disappeared, which suggested that the retrograde conduction from the A-V node to the atria had ceased. With no P wave, the ventricular complex was usually of normal shape, indicating that the ventricles were being driven by the A-V node. Sometimes, however, the shape of the QRS complex varied from beat to beat, with the patterns repeating themselves unpredictably. This suggested that the rhythm was no longer originating in the A-V node, but arose from ectopic foci in the ventricles themselves. At the lowest temperatures these ectopic beats were usually the only ones present. In one heart, however, the A-V nodal rhythm persisted to 5°C, at which point the heart stopped. Ectopic ventricular beats alone were recorded in three hearts at 7.8°, 5° and 3.9° respe tively. In one heart, which showed ecto ventricular beats at high temperatures, irregular, multifocal, ectopic, ventricu beat of one per minute was obtained a short time at 0°C.

Tissue culture

The rate of contraction of cultures heart cells became slower with chillin but the sensitivity to temperature seem to depend more on slight changes in t method of culture than on the species animal from which the explant was tained. Even under the most rigorou controlled conditions we could discov no consistent differences between the sponses of rat and hamster. When the te perature-rate relationships of three se rate foci of contraction in a single expla were found to be markedly different, it came apparent that unknown factors we influencing the response of each focus changes in temperature. If any influen of the donor on the explant existed, it w completely masked by these factors.

General

The heart rates of each species exce grey squirrels and mountain beavers we plotted against temperature using the *A* rhenius equation, which is designed to d scribe the effect of temperature on the ra of simple physicochemical systems. In case was the resulting plot a straight lin In spite of the difficulties in measuring t various components of the EKG, it w quite apparent that the lengthening of t duration of the P wave, the P-R interv and the QRS complex as the temperatu was reduced was typical for each speci studied. When these lengthenings we plotted against temperature, there was characteristic above 10°C which disti guished the hearts of hibernators as group from the hearts of non-hibernato At lower temperatures, however, the inte vals of the hearts of hibernators tended lengthen greatly, while the hearts of no hibernators stopped beating. In the woo chuck, ground squirrel and hamster t P-R intervals showed the greatest lengthe ing in proportion to their original duratio below 7°C, thus confirming the results Dawe and Morrison ('55).

DISCUSSION

Figure 1 shows graphically the phylo-
netic relationships among the various
ecies of rodents used in these experi-
ents. The *Sciuromorpha* is considered to
the more primitive of the two suborders
own here, and the mountain beaver
Aplodontia rufa) is universally agreed to
the most primitive rodent living in the
orld today (Pfeiffer, '56). The grey squir-
l, ground squirrel, woodchuck and chip-
unk are closely related, though the
rmer is separated from the others by
mpson ('45) at the tribal level. Of this
paration he says, "I have moved the
armotini nearer the *Sciurini* because
ese two are surely very closely related
nd perhaps should not be separated even
s tribes." The hamster and cotton rat are
so separated only at the tribal level. Both
elong to the family *Cricetidae*, or "New
Vorld" mice, and are considered more
rimitive than the rat which is a member
f the *Muridae* or "Old World" mice.

The heart rate-temperature curves of the
nimals studied here are typical for each
pecies and could be used as physiological
haracters to separate one species from
nother. However, although the curves are
haracteristic, the responses to changes in
emperature in hearts of closely related
pecies show no obvious similarities which
vould indicate taxonomic affiliations.

Dawe and Morrison ('55) working with
ntact hedgehogs (*Erinaceus*) and arctic
round squirrels (*Citellus parryi*) were
rst to emphasize that there was a break
n the temperature-rate curves of the heart
s the animals entered hibernation. In
hese two species the relationship of heart
ate to body temperature was almost linear
own to 20°C, but below this temperature
he hearts were hyperirritable and con-
inued to beat at lower temperatures than
vould be predicted from extrapolation of
he curves. In the 4 species of hiber-
ators reported here, it was again the break
rom linearity at lower temperatures which
esulted in the hearts from hibernators
ontinuing an organized beat when the
earts from non-hibernators had been
topped by cold. In these *in vitro* experi-
nents the break in the curve was character-
stic of the species, varying from a sharp
change at 12°C in the chipmunk to a gradual curve below 24°C in the hamster. Exact comparisons of *in vivo* and *in vitro* work should not be carried too far since Adolph ('51b) has shown that temperature-rate curves of hamster, rat and cat hearts are not the same under these two conditions.

The shape of the temperature-rate curve may reflect the habits of the hibernator. *Citellus* lives farther north than any other hibernator in this country, and it is possible that a heart which will function at slightly subzero temperatures may have a survival value when the animal is surrounded in its burrow by frozen ground or perma-frost. The Syrian hamster is restricted to warmer climates. If the ambient temperature is reduced to about 0°C while this animal is hibernating, the hamster either increases its metabolic rate and keeps its body temperature above 2.5°C or else it dies in hibernation (Lyman, '48). The normal EKG of the isolated heart ceases at about 2.5°C, suggesting that hibernating hamsters which chill below 2.5°C die of heart failure. The heart in the intact animal would probably not beat at lower temperatures than the isolated heart, for Adolph ('51b) has shown that the opposite is the case in cats and rats. It may be pertinent that the peripheral nerve of the hamster also ceases to function *in vitro* at about 2.5°C so that the animal faces double jeopardy at this body temperature (Chatfield et al., '48).

The hibernating woodchuck also increases its metabolic rate when exposed to near zero temperatures, but the lowest body temperature it can tolerate has not, to our knowledge, been determined. Little is known concerning hibernation in the chipmunk, but the information to date seems to indicate that this animal does not reach the deeply hibernating state of the other three hibernators used in this study (Woodward and Condrin, '45; Panuska and Wade, '58). If the *in vitro* studies are any criterion, it may be predicted that they do not hibernate at deep body temperatures below 5° to 7°C.

The relationship of heart rate to temperature has been studied repeatedly with the hope of finding some general rule

which will clarify the basic problem of the automaticity of the heart, but the results have often been contradictory. A review of the literature shows that the methods of chilling intact, warm-blooded, whole animals, or the isolated hearts, have varied greatly and it is not surprising that the results have differed also, as Brooks et al. ('55, p. 179) have pointed out.

As far as the hearts of hibernators are concerned, the majority of information concerns work on intact animals, either chilled or under conditions of natural hibernation. In this regard, the conditions of the experiment are again of great importance. Dawe and Morrison ('55) have shown that heart rates of animals arousing from and entering into hibernation have very different temperature relationships. In the former case the heart is being driven at near maximum speed for the given temperature (Chatfield and Lyman, '50), while in the latter the heart is being slowed by factors other than temperature (Lyman, '58).

Little work has been done with the isolated hearts of hibernators, but the oftenquoted paper of Endres et al. ('30) on the hearts of two European marmots (*Marmota marmota*) is open to question since both hearts ceased to function at 16°C while the animal is known to hibernate at a body temperature of 5°C. Chao and Yeh ('51) found that the isolated heart of the hedgehog continued to beat at 0 ± 1°C. Hirvonen ('56) showed that the atrium of the hamster ceased contracting between 1.5° and 6°C and found a straight line relationship when plotting the logarithm of the rate as a function of temperature. Our results with hamster hearts are similar to those reported by Adolph ('51a) except that with us the hearts rarely stopped above 6°C.

None of the heart rates of the 7 species which were graphed fit the Arrhenius equation over the whole temperature range, and there is no characteristic break in the Arrhenius plots which can be assigned to the hearts of the hibernators compared to the non-hibernators. If by any chance there is one rate-limiting reaction which controls the automaticity of either group, it is certainly not exposed by this mathematical formula.

Actual hibernation in no way improve the ability of hamster hearts to tolera low temperatures, which is in agreeme with similar data presented on the peripl eral nerve (Chatfield et al., '48). Th hearts of the grey squirrels may have u dergone some adaptation to cold durin the actual experiments, for slow coolin resulted in some sort of rhythmicity lower temperatures. Slow cooling made n difference in the response of the hearts the other 7 species.

Dawe and Morrison ('55) reported th all elements of the EKG lengthened a their hibernators became cooler. The found that a disproportionate lengthenin of the T-P interval accounted for th greater part of slowing of the heart rat The P-R interval lengthened much less tha the T-P interval, but more than the othe components of the EKG. Nardone ('55 working with *Citellus parryi barrowensi* soon confirmed these results. Saraja ('54), using the hibernating hedgehog, ha shown a few months before that the grea est proportional lengthening occurred i the R-R interval, while the spread of th P-R interval was next in magnitude. Sim lar results were reported by Kayser ('57 for several species of hibernators and non hibernators. The present paper furthe confirms that the same order of lengthen ing of the components of the EKG occur in isolated hearts of both hibernators an non-hibernators. In the hibernators, how ever, all intervals except the T-P becom relatively much longer than in the non hibernators, for their hearts continue t beat at low temperatures in which the non hibernating heart cannot function. It i well established that all elements of th EKG lengthen when a heart rate slow without a change in temperature (Brook et al., '55). The reason for this has no been clarified, but the great lengthenin of all elements from P to T wave in the chilled hearts of hibernators must be du to the low temperature, since the heart of non-hibernators beating at slower rate at higher temperatures do not have as grea a lengthening of these elements.

The evidence indicates that some part of the hearts of the species tested here ar more sensitive to cold than other parts, an that this sensitivity varies from species t

ecies. The suggestion that the A-V node mammalian hearts is most sensitive to ld (Brooks et al., '55; Lyman and Chat-eld, '55) does not seem to be universal. the cotton rat, chipmunk and hamster ilure of the A-V node is indicated since e P wave persisted after ventricular activ-y had ceased. In general, the hearts of e mountain beaver, rat and woodchuck owed no electrical activity once the nor-al sequence of depolarization had ceased, ough in the latter two species the A-V ode must have acted occasionally as pace-aker and conduction must have occurred hen the ventricles alone continued to eat at low temperatures.

Our interpretation of the EKGs of the rey squirrels suggests a range of sensi-vities to cold for different parts of the eart. In this case the atria appear to be ast capable of functioning in cold, the -V node next, and the ventricles the most ardy. The whole heart of the ground quirrel is apparently capable of a slow ormal sequence of depolarization at least s low as −1°C.

Although his study did not concern hi-ernation, Adolph ('51a) showed that the olated hearts of infant hamster, rat and ouse ceased to beat at the same tempera-ure as isolated adult hearts, and that both nfant and adult hamster hearts could olerate lower temperatures than the hearts f rats and mice. In our experiments with ssue culture the species of the donor did ot appear to influence the response to emperature in a simple aggregate of heart ells. The fact that slight changes in the nethod of culture did affect the responses f the explants emphasizes the importance f environment on developing tissues in neir responses to temperature in later life.

The concept that the ability to function t low temperatures is primitive is not orne out by the evidence. The heart of ne most primitive rodent (mountain eaver) is as sensitive to low temperatures s is the heart of a species from the most nodern group of rodents (Norway rat). Actually there seems to be no obvious rea-on why the hearts of various species of non-hibernating mammals should vary in heir sensitivity to cold, since their body temperatures probably do not change more than 3° or 4°C during their entire adult life.

On the other hand, the ability of the hearts of hibernators to function at low temperatures has an obvious survival value, and it is not surprising that this capability is universal with them. It is peculiar, however, that species closely related to the hibernators do not have this ability. This suggests that hibernation occurred here and there in the mammals as the ecological situation required with no regard for the phylogeny of the species. If this is the case, it would be remarkable if hibernation were precisely the same in every mammalian group, since it may have occurred independently again and again. This forms an obvious explanation for the variations from species to species in the physiology of hibernation.

SUMMARY

This research is an attempt to clarify the fundamental differences in response to low temperatures betwen the tissues of hibernating and non-hibernating rodents. Isolated, perfused hearts from 4 species of hibernators (*Citellus, Marmota, Tamias* and *Mesocricetus*) and 4 species which do not hibernate (*Sciurus, Rattus, Aplodontia* and *Sigmodon*) were exposed to progressively colder temperatures until the hearts stopped beating. The hearts of each species showed a typical rate-temperature curve, which was the same during chilling or rewarming.

All the hearts of animals which could hibernate continued an organized beat at lower temperatures than the hearts of non-hibernating animals. The temperature-rate curves of hibernators were linear at high temperatures, but became curvilinear as the temperature was lowered, so that the hearts beat at lower temperatures than would be predicted if the upper part of the curve were extrapolated to 0°C. On the other hand, the temperature-rate curves of the non-hibernating rodents were almost linear over the whole temperature range. Only in the grey squirrel (*Sciurus*) did variations in the rate of chilling effect the response. In this species the heart usually

stopped at 13–16°C, but an irregular, ectopic, ventricular beat could sometimes be elicited down to nearly 0°C if the chilling was very slow.

The ability to beat at low temperatures varied among the various species of hibernators. The heart of the ground squirrel (*Citellus*) continued to function at −1°C while the organized beat in the heart of the chipmunk (*Tamias*) stopped at about 7°C. This variation showed correlation with the known habits of the various genera. Hearts removed from hamsters during hibernation were no more capable of functioning at low temperatures than were the hearts removed from active animals.

Hibernators and non-hibernators with close phylogenetic affinities showed no obvious similarities in the temperature-rate curves of their hearts. None of the curves fitted the Arrhenius equation over their whole temperature range. There was no evidence that tolerance to cold was a primitive characteristic, for the heart of the most primitive living rodent (*Aplodontia*) was as sensitive to cold as the heart of a "modern" rodent (*Rattus*).

At temperatures where they could be compared, the lengthening of the various components of the EKG with chilling were similar in hibernators and non-hibernators. Except for the T-P interval, the lengthening of the P-R interval was proportionally greatest in both groups. Below 10–15°C, when the hearts of the non-hibernating rodents had stopped, all elements of the EKG in the hibernating rodents were greatly lengthened. Although conduction at the A-V node usually failed first as the hearts were chilled, this was not invariably the case in every species.

It is concluded that, among rodents at least, the adult hearts of species which hibernate are peculiar in their ability to continue an organized beat at low temperatures and that this ability does not exist in closely related species which are incapable of hibernation. We were unable to demonstrate that this characteristic was typical of the cells themselves, for the contraction during chilling in tissue cultures of the hearts of infant rat and hamster seemed to depend on other factors than the species of the donor.

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Chloride Movements in Cerebral Cortex After Circulatory Arrest and During Spreading Depression[1]

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Asphyxiation of the cerebral cortex causes after a latency of several minutes a sudden increase of the cortical impedance (Van Harreveld and Ochs, '56) which is accompanied by the development of a surface negativity with respect to an indifferent electrode (Leão, '47, '51; Van Harreveld and Stamm, '53; Van Harreveld and Ochs, '56; Van Harreveld, '57). After the rapid increase, the cortical impedance continues to rise at a lower rate. Spreading depression is characterized by similar features. The depression of the electrocorticogram is accompanied by cortical negativity which is usually followed by surface positivity (Leão, '47, '51; Marshall et al., '51; Van Harreveld and Stamm, '51; Bureš, '54). The cortical impedance is raised markedly during spreading depression (Leão and Ferreira, '53; Freygang and Landau, '55; Van Harreveld and Ochs, '57). The rapid impedance increase after asphyxiation caused in the rabbit a decrease in cortical conductivity (1/R) up to 30–35%; during spreading depression the decrease in conductivity was seldom more than 15%, often considerably less.

From impedance measurements of cell suspensions Fricke ('24) and Cole ('40) concluded that the value of this parameter is mainly determined by the amount of extracellular ions. These findings are applicable to a tissue which also consists of cellular elements and an intercellular fluid or substance. It was postulated therefore that the large increases of cortical impedance observed after asphyxiation and during spreading depression are due to a movement of ions from the intercellular space into the cells and fibers of the cortex. The latter structures are surrounded by membranes of relatively high resistance which make the intracellular ions unavailable for the transport of the measuring current. A movement of ions from the extra- into the intracellular compartment will be recorded therefore as an impedance increase (Van Harreveld and Ochs, '56). To maintain osmotic equilibrium the ion transport must be accompanied by a transport of water from the extra- into the intracellular space. The predicted swelling has indeed been observed in perikarya and apical dendrites after circulatory arrest (Van Harreveld, '57) and in apical dendrites during spreading depression (Van Harreveld, '58).

Sodium and chloride are probably the most abundant extracellular ions. An increase in the sodium chloride content of the intracellular compartment can therefore be expected from the postulated ion movements. Since chloride ions are the easier to detect, their distribution in the cortex after asphyxiation and during spreading depression was compared with the position of these ions in the normal cortex.

METHOD

In order to determine the position of the chloride in the tissue it had to be precipitated before it could diffuse away from the position it held under the experimental conditions to be investigated. The chloride was precipitated with a silver salt. The resulting silver chloride was made visible by reduction to its subhalide by exposure to light. Gersh ('38) kept the chloride ions in their position by quick freezing followed by lyophylyzing. However, the necessary wetting of the dry tissue with the silver solution could result in movements of the

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chloride ions. It was preferred to place the quickly frozen tissue at a temperature well below 0°C in an alcohol-water-silver nitrate solution of a freezing point lower than the temperature in the refrigerator (substitution fixation). The ice in the tissue was slowly dissolved and replaced by the silver solution which precipitated the chloride as silver chloride. The tissue should be kept below its eutectic point to keep the ions in the location they held at the moment of freezing. At temperatures above −10°C the cortical tissue is relatively soft and consists obviously of a mixture of ice crystals and a concentrated solution of the tissue solutes. For practical reasons a temperature of −25°C was chosen for the treatment with the silver nitrate solution. This temperature is below the eutectic points of potassium– and sodium chloride solutions, but still above that of a calcium chloride solution. However, at −25°C the tissue is very hard and contains undoubtedly very little free water. After considerable experimentation a 90% alcohol solution saturated with silver nitrate was chosen to precipitate the chloride in the tissue. Due to its high alcohol content this solution has a freezing point well below −25°C, while the 10% water it contains keeps a sufficient amount of silver nitrate in solution (about 2.5% at −25°C). This solution dissolved the ice in the tissues very slowly. The alcohol and silver nitrate penetrated 1 to 1½ mm into the cortex during the time (three days to one week) the tissues were left in the refrigerator. The initial freezing of the cortex had to be done at the highest rate possible to prevent the formation of large ice crystals. Used was isopentane cooled to its freezing point (−160°C) with liquid nitrogen.

The experiments were performed in the following way. In rabbits and cats the trachea and the jugular vein were cannulated and the cortex was exposed under ether narcosis. Artificial respiration was given and Squibb's Intocostrin (5–10 U./kg bodyweight) was injected intravenously. In rats the cortex was exposed and the trachea was cannulated under light ethyl carbamate narcosis. Silver chloride-plated silver wires mounted on springs were placed on the cortex to lead off electrocorticograms and to measure cortical impedance as described previously (Van Harreveld and Ochs, '56). Spreading depressions were elicited by direct current stimulation (4–6v). In rats the head was severed from the body and quickly immersed in cooled isopentane at the desired moment. After a few minutes the head was placed in isopentane at a temperature of −30 to −35°C. Three minutes later the isopentane was rinsed off with alcohol of the same temperature, then the head was placed in the alcohol-water-silver nitrate solution at −25°C. In larger animals (rabbit, cat) a cup was formed by sewing the skin edges of the wound exposing the brain, to a steel ring. At the moment of choice the skin cup was filled rapidly with cold isopentane. Then the head was severed from the body after which it was treated as described for the rat.

After three days to one week in the alcohol-water-silver nitrate solution at −25°C the desired cortical region was isolated, washed for 4–5 hours in 70% alcohol at about 0°C, dehydrated and embedded in paraffin. Mounted sections 10 μ thick either unstained or after staining with gallocyanin, were exposed for 10 to 15 minutes to sunlight to reduce the silver chloride.

The method was tested on striated muscle, a tissue in which the extracellular position of most of the chloride is generally accepted (Eggleton et al., '37; Boyle et al., '41; Mokotof et al., '52). Figure 5 shows a cross section of the m. rectus abdominus of a rat treated in the way described above. The muscle fibers appear light, and are surrounded by dark material, the reduced silver chloride. This photomicrograph shows that indeed most of the chloride in the muscle has an extracellular location. Manery et al., ('38) found that the chloride content of connective tissue is relatively high. In agreement with this the fascia shown in figure 5 is dark.

RESULTS

Chloride movements after circulatory arrest

A. Experiments with rats. After removal of the dura two leading off and two impedance electrodes were placed on the

rtex as shown in figure 1A. In 10 rats e circulation was arrested by cutting the bdominal aorta. Table 1 shows the cor- cal conductivity (expressed in mhos 10^5) before circulatory arrest. The fast mpedance rise usually started after one two minutes. About 2½ minutes later, hen the rate of the impedance increase as dropping off markedly, the head was evered from the body and frozen. At that me the mean decrease in conductivity as 43%.

In 10 control experiments the head was evered and frozen without cutting the aorta. It will be shown that spreading depression causes similar histochemical changes as asphyxiation. By recording the electrocorticogram and measuring the cortical resistance it was possible to make sure that no spreading depression was in progress at the moment of freezing, which would have made the preparation useless as a control. The location of the slices of cortex used for microscopic examination is indicated in figure 1A.

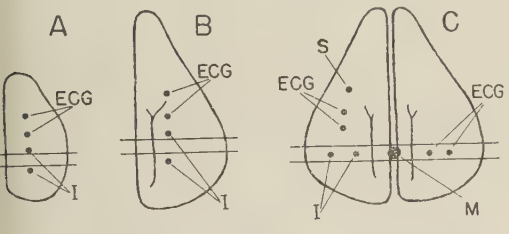

Fig. 1 A and B show the electrode placements for rats and rabbits in experiments in which the effect of circulatory arrest was investigated. The same placement was used in the control experiments. C gives the electrode placements in experiments on spreading depression in rabbits. Electrode pairs leading off electrocorticograms are indicated by ECG, those used for impedance measurements are marked I. S indicates a stimulating electrode. M shows the marker on the sagittal bone ridge which indicates the position of the impedance electrodes in the spreading depression experiments. The slices of cortex removed for the microscopic examination are indicated by the horizontal lines.

Figures 3A and B show typical sections of control cortex and of cortex frozen after the impedance change had taken place. Such preparations on macroscopic inspection looked alike, both showing a brown color of about the same intensity. This is to be expected since the total amount of chloride should be the same in both kinds of preparations. On microscopic examination the distribution of the chloride was quite different, however. On the control slides it was rather evenly distributed. Only the pia mater, the walls of blood vessels and the blood appeared as darker structures. Blood plasma contains considerable amounts of chloride (about 100 meq/l), and connective tissue has been reported to contain an even higher concentration of chloride (Manery et al., '38) which accounts for the darkness of these structures. The apical dendrites and the perikarya appeared light yellow, sometimes of the same shade of yellow as the surrounding tissue, often slightly darker (fig. 7A). The boundaries of the larger

TABLE 1

Rats

Cortices after circulatory arrest						Control cortices		
No.	Conductivity[1] before circ. arrest	Start impedance change	Moment of freezing	% Decrease in conductivity	Evaluation of preparation	No.	Conductivity[1]	Evaluation of Preparation
1	33	1′10″	4′30″	42	+++	1	45	0
2	28	2′00″	4′00″	34	++	2	37	0
3	29	2′00″	4′30″	41	+++	3	37	0
4	32	0′50″	3′10″	42	++	4	32	0
5	29	1′40″	3′50″	53	+++	5	30	0
6	37	1′30″	4′00″	35	++	6	27	0
7	34	0′50″	3′50″	56	+++	7	31	0
8	32	2′05″	4′10″	44	+++	8	25	0
9	26	2′00″	3′50″	40	+++	9	32	0
10	41	1′50″	3′40″	42	+++	10	28	0
Means	32	1′35″	3′55″	43			32	

[1] Expressed in mhos $\times 10^5$.

dendrites were formed by dark lines which made them stand out clearly against the background (figs. 7A and 8A). This suggests the presence of yellow-brown subhalide near the surface of the dendrite. The thinner apical dendrites usually did not show the surface darkening clearly and were therefore often hard to recognize. The tissue in between the apical dendrites had a foamlike appearance, the walls surrounding the cavities were formed by the yellow-brown material (figs. 7A and 8A). This tissue did not show any recognizable anatomical structures.

The microscopic features of the cortex frozen after the rapid impedance change had taken place were quite different. Most evident was the presence of brown often granular material in apical dendrites which made them the outstanding feature in these preparations (fig. 3B). There was always a considerable difference between the individual apical dendrites, some appearing very dark, others brown and some only slightly more yellow than the dendrites in the control cortices. This lack of uniformity in the apical dendrites is well demonstrated in figure 7B. The cytoplasma of the perikarya was usually slightly more yellow than in the control preparations. However, the region close to the origin of the apical dendrites was often considerably darker. The basal dendrites did not differ from those in the control cortices. Branches of the basal dendrites could not be distinguished, neither could axons be recognized. The space in between the neuronal elements appeared to be lighter than in the control preparations (figs. 3B, 7B). In most preparations the molecular layer was poorly stained (fig. 3B). This may be due to the treatment of the cortex with alcohol to rinse off the isopentane. The ice and chloride in the most superficial layer of the cortex may have been removed in this way. In those preparations in which the molecular layer was well stained the branching of apical dendrites could clearly be distinguished. Figures 7A and B which show apical dendrites before and after the rapid impedance change, demonstrate the swelling of apical dendrites during the conductivity drop described previously (Van Harreveld, '57).

The staining did not extend throughou the entire thickness of the cortex. A gall cyanin stained preparation of rat corte is shown in figure 3C. The two granula layers (II and IV) can be distinguishe A comparison with figure 3B indicates tha the staining may reach into layer 5.

To allow a semiquantitative evaluatio of the changes in the position of the chlo ide ion, a scale was devised in which th average control preparation was desig nated as 0 and the preparations wit the darkest apical dendrites as +++. I between these, two additional steps wer distinguished completing the scale: 0, + ++ and +++. Table 1 shows the evalua tion of all the rat preparations by compar son with this scale. The control prepara tions were all evaluated as 0; of the exper mental cortices 7 were estimated as +++ and three as ++. The histological dif ferences between cortices frozen befor and after the rapid impedance change ha taken place were thus found very consis tently.

B. Experiments with rabbits. A simi lar series of experiments as describe above for the rat was performed on rab bits. The placement of the electrodes re cording the electrocorticogram and the im pedance changes are shown in figure 1B The 10 control cortices of this series wer frozen while the circulation was intact The electrocorticograms and the imped ance measurements proved the absence o spreading depression. In 10 other prepa rations the cortices were frozen after the rapid impedance increase had developed and its rate was going down. Table 2 shows that the impedance change usually started between two and three minutes after cutting the aorta. The cortex was frozen about two minutes later. The mean decrease of cortical conductivity during this period was 34%. After the head had been treated with the alcohol-water-silver nitrate solution at −25°C the slice of cortex indicated in figure 1B was embedded

Figure 4A and B are sections of a control cortex and of a cortex frozen after the rapid impedance change. They show similar differences as found in the rat under the same conditions. In the control preparations the colored material was more or less evenly distributed. The apical

TABLE 2
Rabbits

Cortices after circulatory arrest						Control cortices		
No.	Conductivity[1] before circ. arrest	Start impedance change	Moment of freezing	% Decrease in conductivity	Evaluation of preparation	No.	Conductivity[1]	Evaluation of Preparation
1	34	3′10″	5′00″	38	+++	1	34	0
2	43	1′50″	3′30″	35	++	2	32	0
3	27	2′10″	5′30″	39	+++	3	35	0
4	34	2′10″	4′00″	32	++	4	32	0
5	33	2′00″	4′30″	27	++	5	34	0
6	26	2′00″	4′40″	46	+++	6	34	0
7	34	2′30″	4′00″	35	+++	7	27	0
8	36	2′50″	4′10″	28	++	8	33	0
9	29	2′00″	5′20″	31	+++	9	32	0
10	28	2′10″	4′30″	29	++	10	35	0
eans	32	2′15″	4′30″	34			33	

[1] Expressed in mhos $\times 10^5$.

endrites and the cytoplasma of the nerve ells were often somewhat more yellow han the surrounding, but in some prepara- ons they were of the same shade. fter the rapid impedance change had aken place apical dendrites again became he most obvious feature of the prepara- ion, being brown and often containing ark granular material. There were con- iderable differences in the individual den- rites, some being very dark, others less so nd some not much darker than in the ontrol preparations. The cytoplasma of he nerve cells appeared somewhat more ellow than in the control sections. No arkening of apical dendrites was ob- erved. The material in between the apical endrites became lighter.

Figure 8A shows some apical dendrites nd nerve cells at high magnification in a ontrol cortex. The dark boundary lines f the apical dendrites and the foamlike tructure of the tissue in between the den- drites can be easily recognized. Figure 8B s a section of a cortex frozen after the apid impedance change had taken place. The black material in one of the dendrites s irregularly distributed. In other den- drites it is situated near the surface.

The rabbit cortex is thicker than that f the rat. A comparison of figure 4A and 3 with a gallocyanin stained section (4C) hows that the silver ions did not pene- rate much deeper than the second granu- ar layer (IV).

The rabbit preparations were evaluated sing the scale described for the rat. The results are given in table 2. All the control preparations were designated as 0. Of the experimental preparations 5 were evaluated as +++, and 5 as ++. Again a very consistent difference was found between the controls and cortices frozen after the rapid impedance change had occurred.

C. Experiments with cats. In a small series of cats sections of cortices frozen before and after circulatory arrest were studied. The electrodes leading off the electrocorticogram and those measuring the impedance were placed on the gyrus suprasylvius medius which was later used for the anatomical study. The cortex was sectioned at right angles with the length direction of the gyrus. The sections of 4 control experiments in which the cortex was frozen while the circulation was intact showed the same features as the control preparations of rats and rabbits. They were more or less uniformly yellow, no particular neural structure standing out. Apical dendrites and nerve cells could be identified mainly by their typical location and shape.

In 4 cats the aorta was severed causing an impedance increase which began 2½ to 6½ minutes after the start of the circulatory arrest (table 3). A few minutes later when the impedance increase slowed down, the brain was frozen. The sections of these cortices showed like those of rats and rabbits the presence of brown material (often in distinct granules) in apical dendrites. The amount of this ma-

TABLE 3
Cats

Cortices after circulatory arrest						Control cortices		
No.	Conductivity[1] before circ. arrest	Start impedance change	Moment of freezing	% Decrease in conductivity	Evaluation of preparation	No.	Conductivity[1]	Evaluation of Preparation
1	29	2′20″	4′30″	27	+++	1	25	0
2	22	6′20″	9′30″	43	+++	2	21	0
3	40	3′10″	4′30″	27	++	3	38	0
4	34	5′00″	7′10″	32	++	4	19	0

[1] Expressed in mhos $\times 10^5$.

terial varied considerably in the individual dendrites. The area in between these structures was lighter than in the control preparations. The cytoplasma of the nerve cells had darkened very little.

The preparations were evaluated using the scale described for the rat. All the control preparations were designated as 0. From the asphyxiated cortices two were evaluated as +++ and two as ++.

Chloride movements during spreading depression

The potential and impedance changes recorded during spreading depression and after circulatory arrest are quite similar. Furthermore, the apical dendrites increase in diameter under the same circumstances. A chloride movement like the one after cortical asphyxiation can therefore be expected during spreading depression. However, since the impedance increase accompanying the latter was always considerably smaller than the asphyxial changes, the ionic movement could well be less pronounced.

In rabbits the two hemispheres were exposed leaving a narrow bone ridge over the sagittal sinus intact. The parasagittal sulci were exposed and as much of the retrosplenial areas as feasible. A stimulating electrode was placed on a frontal region of one hemisphere. A few millimeters caudal a pair of electrodes were placed leading off an electrocorticogram, and still more caudal were located electrodes for the measurement of cortical impedance. The position of the latter was marked on the bone ridge. An electrocorticogram was recorded from the opposite hemisphere with a pair of electrodes placed symmetrically with the impedance electrodes. The placement of the electrode used is shown in figure 1C. Spreading depression was elicited by applying direct current (4–6v) to the stimulating electrode against an indifferent one. Figure 2 shows the results of such an experiment. The first manifestations of spreading depression were typical changes in the electrocorticogram followed by an increase in impedance recorded between the more caudal pair of electrodes. When the impedance had reached a maximum and started to decline the cortex was frozen. The unchanged electrocorticogram taken from the electrode pair on the heterolateral control cortex showed that no spreading depression was in progress there. After treatment with the alcohol-water-silver nitrate solution two slices of cortex, the location of which was indicated by the mark on the bone ridge, were removed for microscopic examination (fig. 1C). In such a slice of experimental cortex the changes typical for spreading depression should be at their maximum. Care was taken to include the retrosplenial area on both sides.

Table 4 gives the results obtained in 15 preparations. The change in conductivity caused by spreading depression varied from 3 to 18% in individual experiments, the mean being a decrease of 8.5%.

The sections of the cortex on the control side showed the features typical for the normal oxygenated cortex. The chloride was more or less evenly distributed. The apical dendrites were light yellow not standing out against their surrounding. On the experimental side frozen at the height of the spreading depression the apical dendrites were darker than the surrounding tissue (fig. 6). In some preparations the darkening of the apical den-

tes was marked, in others they were ly slightly more yellow than on the con- l side. The scale described above was ed for the evaluation of these prepara- ns. Table 4 shows that the estimates were between ++ and 0–+. In the latter preparations the apical dendrites were just noticeably darker than those in the control sections of the heterolateral cortex. A comparison of this evaluation with the

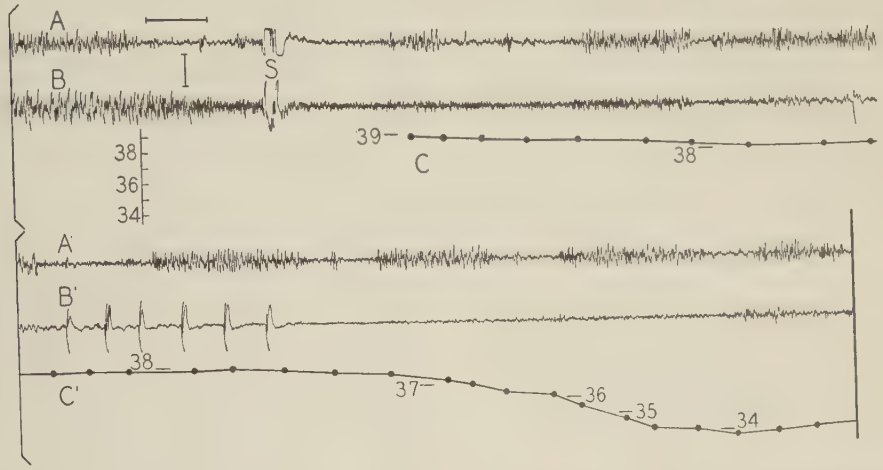

Fig. 2 Shows the electrocorticograms and impedance changes during a spreading depression experiment recorded with the electrode arrangement indicated in figure 1C. The corticogram A and A′ was taken from the control hemisphere. It shows periods of arousal but there are no indications of spreading depression. The DC stimulus artifact eliciting the spreading depression on the heterolateral hemisphere is indicated by S. The electrocorticogram B and B′ was taken from this hemisphere. It is also aroused, but shows in addition a clear-cut depression mixed with some spike activity. The conductivity changes (expressed in mhos $\times 10^5$) are indicated by C and C′. The conductivity drop started after the spikes and depression in the electrocorticogram B and B′ had developed. After the conductivity drop had reached its maximum the cortex was frozen (indicated by the vertical line). A time period of 10 sec. is indicated by the horizontal line. The vertical calibration line indicates 1 mv for both electrocorticograms.

TABLE 4

Spreading depression

	Experimental cortices				Control cortices
No.	Conductivity[1] before SD	Conductivity[1] during SD	% Decrease in conductivity	Evaluation of preparation	Evaluation of preparation
1	30	26	13	++	0
2	40	35	13	++	0
3	39	36	8	+	0
4	35	33	6	0–+	0
5	30	26	13	+	0
6	40	34	15	++	0
7	40	38	5	+	0
8	43	40	7	+	0
9	39	32	18	++	0
10	41	39	5	0–+	0
11	37	34	8	++	0
12	45	43	4	0–+	0
13	39	37	5	+	0
14	33	32	3	0–+	0
15	34	32	6	+	0
Means	38	34	8.5		

[1] Expressed in mhos $\times 10^5$.

changes in conductivity caused by spreading depression shows that there exists a crude correlation between these two values. For the following reasons no more than a crude correlation can be expected. The magnitude of the impedance change will depend not only on the ionic movement in the cortex, but also on the position of the front of spreading depression with respect to a line connecting the impedance electrodes. The largest impedance change will be recorded when this front reaches the resistance electrodes simultaneously. If this is not the case the chloride movement may be more pronounced than could be expected from the impedance rise. On the other hand, due to shrinking during fixation the sections observed may not have been located exactly under the impedance electrodes. In such sections the chloride movement will be smaller than is indicated by the conductivity drop.

As mentioned above the retrosplenial area was included in the microscopic preparations. This area is of special interest since it is not normally invaded by spreading depression (Leão, '44; Van Harreveld and Bogen, '56). Figure 9A shows a preparation which demonstrates this fact. It shows the parasagittal sulcus in which are situated some large blood vessels. The sulcus forms the lateral boundary of the retrosplenial area which is situated to the right in figure 9A. The section is from a cortex frozen at the height of spreading depression. To the left of the sulcus the apical dendrites, which were cut at a slight angle with their length direction, are dark. This change is most pronounced in the superficial layers of the cortex. To the right of the sulcus the cortex shows the features of the control cortex. It is obvious from this figure that spreading depression halts sharply at the parasagittal sulcus.

It has been found that the asphyxial potential change does occur in the retrosplenial area, although it may start there somewhat later than in the rest of the cortex (Van Harreveld and Bogen, '56). Figure 10 shows the cortex on both sides of the parasagittal sulcus in a preparation frozen after the asphyxial changes had taken place. It shows the darkening of the apical dendrites typical for such preparations, which is present in both the r trosplenial area and in the cortex lateral the parasagittal sulcus.

The nature of the yellow-brown materi in the cortical sections

It has been assumed that the yello brown material in the sections of cortic tissue is silver chloride reduced by lig to its subhalide. This photoreaction typical for the silver halides. Chloride by far the more abundant of the halides i the cortex. The colored material after e posure to light could therefore well be r duced silver chloride.

A number of reactions for silver chlorid were carried out. Treatment of prepar tions before exposure to light with a 10% sodium thiosulfate solution dissolved th photosensitive compound. So did ammoni and concentrated hydrochloric acid. A these reagents form complex compound with silver chloride which are soluble.

Feigl ('43) described a test for silve chloride in which potassium nickel cyanid reacts with silver halide to form nick cyanide. The latter produces enough ni kel ions to form with dimethylglyoxime red insoluble precipitate. Indeed when thi reaction was carried out on the slide th sections turned red. On microscopic ex amination the histological features wer found faintly in red, part of the nickel di methylglyoxime had crystalized in lon crystals, however. According to Feigl thi reaction is positive with a number of sligh ly soluble silver salts of which only silve chloride can be expected to be formed i the tissue.

It was furthermore attempted to fin quantitative support for the postulate tha the yellow-brown material in the section is formed from silver chloride. The corte contains about 35 meq. of chloride/k (Manery, '52). Allowing for shrinking dur ing the alcohol fixation and embedding a cortical section 10 μ thick would contai about 0.05 microequivalent of chloride cm^2. A solution containing one micromol of silver nitrate/cm^3 was mixed with a equal amount of a 5% gelatin solutio containing an adequate concentration o sodium chloride to precipitate the silve ions. The solution was cooled and 1 mm thick plate was cut from it, whic

s exposed to light. Such a plate contains
: cm^2 0.05 micromole silver chloride,
e same as in 1 cm^2 of a 10-μ thick cor-
al section. The dispersion of the silver
loride precipitated in this way is such
at it produces after exposure to light a
nilar yellow-brown color as the cortical
ctions. A comparison of the tissue sec-
ns and the gelatin plate showed them
be of an approximately equal intensity
brown. This indicates that the amount
chloride in the tissue is roughly sufficient
account for the yellow-brown material
the sections.

The treatment of the cortex with alcohol
d silver nitrate is reminiscent of some
the silver staining techniques for ner-
us tissue, for instance those of Cajal
28). It can be shown in the following
ay that the staining as used in the pres-
t investigation is an entirely different
ocedure. Cortical tissue was fixed in
cohol and washed with distilled water.
rt of it was rapidly frozen and treated
actly as described under method. After
posure to light no photosensitive ma-
rial was found to be present and the sec-
ns appeared colorless. This is consistent
th the postulate that this method indi-
tes the presence of chloride, which will
leached out by washing the alcohol fixed
ssue with distilled water. Silver ions do
ot seem to form photosensitive com-
unds with the proteins at the low tem-
rature (−25°C) used in these experi-
ents. The rest of the fixed and washed
rtical material was stained with one of
e Cajal procedures, resulting in prepa-
tions typical for this technique.

Although it seems likely that most of the
llow-brown material found in the prepa-
tions is formed by the reduction of silver
loride, it seems possible that silver ions
rm photosensitive compounds with other
nstituents of the cortex. They could be
recipitated as organic silver compounds,
lfate, phosphate or carbonate. The addi-
on of phosphoric acid (Gersh, '38) or 1%
itric acid (Gomori, '52) to the alcohol
ater solution will prevent the precipita-
on of silver phosphate and carbonate.
reparations treated with the silver solution
ntaining phosphoric acid or 1% nitric
id showed the typical coloring after ex-
osure to light, but were somewhat lighter
than the preparations treated with a neu-
tral silver solution. These preparations
showed the same distribution of yellow-
brown material in control and asphyxiated
cortices and in cortices frozen during
spreading depression as described above
for preparations treated with neutral silver
solution. These observations indicate that
probably other ions, perhaps bicarbonate
and phosphate ions, participate in the elec-
trolyte transport. It will be understood that
when in this paper chloride transport is
mentioned, it will include these undefined
electrolytes which seem to be transported
in the same way as the chloride.

DISCUSSION

The experiments reported above show
that after circulatory arrest and during
spreading depression an intracortical trans-
port of chloride ions takes place. With the
histochemical method used it was found
that chloride accumulates especially in
apical dendrites and that it disappears
from the tissue in between these struc-
tures. The asphyxial chloride transport
has been studied in this investigation only
during the rapid impedance increase. The
rapid rise is followed by a slower imped-
ance increase during which the chloride
transport may continue at a reduced rate.

After circulatory arrest (and during
spreading depression) not only ions but
also water enters the neuronal elements
causing an increase of about 30% in the
diameter of the apical dendrites (Van
Harreveld, '57). The water and electro-
lytes which move into the apical dendrites
may originally have been part of the inter-
cellular fluid of the cortex. The intercellu-
lar space in the brain has been estimated
from its chloride content as about 30%
of the total volume on the assumption that
all the chloride is extracellular (Manery
and Hastings, '39, Manery, '52, Holmes
and Tower, '55). Allen ('55) estimated
the "ferrocyanide" space in brain tissue
slices as 16%, the "insulin" space as
14.5%. According to these estimates there
are considerable amounts of water and
chloride readily available for the asphyxial
transport. However, electron microscope
studies of the cortex seem to indicate that
the neuronal and glial cells and fibers are
situated in such close proximity that hard-

ly room for significant amounts of intercellular fluid is left (Dempsey and Wislocki, '55; Schultz et al., '56; Luse, '56; Schultz et al., '57; Dempsey and Luse, '58). The remark was previously made (Van Harreveld, '57) that since tissue resistance is mainly a function of the amount of intercellular ions such a concept of the cerebral cortex is hard to reconcile with the relatively low specific resistance (222 ohms) of the cortex determined by Freygang and Landau ('55). Only by assuming that the surface membrane of glial elements is readily permeable for ions could the low specific resistance and the asphyxial ionic movement be reconciled with the electron microscope data. With such a surface membrane, the glia could hardly maintain an ionic composition different from that of the intercellular fluid, however, and the combined volume of the glial elements would have the value of an "extracellular" space for the neuronal elements. Another possibility is that the scarcity of extracellular space in electron micrographs of the cortex is caused by the procedures used in preparing the tissue for electron microscopy and also by the transport of ions and water described above. Since cerebro-spinal fluid contains very little protein (0.02%) and if, as has been claimed (Wallace and Brodie, '39; Weir and Hastings, '39; Manery, '52), this fluid is a fair sample of the intercellular fluid of the brain then it is obvious that after removal of water and solutes very little material is left to fill the intercellular spaces, which may then collapse.

The apical dendrites in sections of cortex frozen after the rapid asphyxial impedance increase were not uniformly dark, some contained a large amount of brown material, others less and some were only slightly more yellow than the dendrites in control preparations. This might be due to a quantitative difference in the processes which lead to the ion movement in the various dendrites. Another possibility is that these processes were the first to start in the darkest dendrites and that at the moment of freezing they had only just begun in the lighter ones. Furthermore the dendrites in which the ion movement starts early would have first call on the ions in a limited amount of intercellular fluid. The perikarya darkened v little during the rapid impedance rise. T mechanisms suggested to explain lightly stained apical dendrites could a account for the slight changes in perikarya. There is no indication that electrolyte movement takes place into ba dendrites. The processes leading to the transport seem to be restricted to neuro

In sections of control cortices the api dendrites appeared in approximately same shade of yellow as the rest of section. The boundaries of the larger d drites were formed by brown lines. In t gentially sectioned preparations, in whi the apical dendrites are cut at right ang the chloride was present near the d dritic surface as a thin circular line. T chloride in the dendrites of asphyxia cortex cut in this way was also often pr ent in a peripheral location as a heav circle. In the darkest dendrites howe chloride was sometimes also observed granules in a more central position. T tissue in between the apical dendrites h a foam-like appearance, the walls of t cavities being formed by yellow-brown m terial.

The question has to be consider whether these features reflect anatomic structures, or that they are caused by i formation in the cortex. It is unlike that the cooling of the cortex as practic in this investigation is sufficiently fast cause vitreous freezing. Ice formati can thus be expected during which t solutes including the chloride will be c centrated in the fluid between the i crystals. This distribution then will fixed by the subsequent alcohol treatme Such a process may account for the foa like appearance of the tissue in betwe the apical dendrites. Eccles ('57) c cluded from an equilibrium potential f chloride ions in motoneurones of 70 m that there is 14 times less chloride in t motoneurone than in the outside flui The chloride concentration in cortical ne rones may be of a comparable magnitud It seems conceivable that ice crystals d veloping inside an apical dendrite for a central core of ice, pushing the solut toward the periphery. The thin da ring in tangentially cut preparations control cortices may thus represent t

mall amount of chloride which can be ex-ected to be present in these structures. lso other electrolytes like bicarbonate and hosphate in the apical dendrites may ontribute to the ring. The same mecha-ism may explain the peripheral position f chloride in the dendrites of asphyxiated ortex. When much chloride is present his mechanism may be disturbed result-ng in the presence of black granules in a nore central position.

Similar changes as observed after cir-ulatory arrest have been found during spreading depression. In general the ion ransport was less pronounced as is com-mensurate with the smaller impedance changes accompanying this phenomenon. These observations emphasize the essen-ial similarity of the processes which un-derly both the asphyxial changes and spreading depression. This was already in-dicated by the similarity in impedance and slow potential changes and by the increase in the diameter of apical dendrites which is characteristic for both phenomena.

It has been postulated that the ion trans-port after circulatory arrest and during spreading depression is caused by an in-crease in permeability of the neuronal membrane for sodium ions and perhaps also for potassium and chloride ions (Van Harreveld and Ochs, '56). Since the mem-brane is normally moderately permeable (and may become more permeable) for po-tassium and chloride, a Donnan situation would be created in this way in which the membrane is permeable for three ions (the two principle extracellular ions, so-dium and chloride and the principle intra-cellular cation, potasssium) but would re-main impermeable for the intracellular or-ganic anion. This will result in a move-ment of sodium chloride from an inter-neuronal space into the neuronal elements. Osmotic equilibrium will be maintained by a water transport into dendrites and nerve cells. This will prevent the building up of concentration differences between the in-side and outside of the nervous elements which would be necessary to arrive at a Donnan equilibrium. Theoretically the movement of water and ions will proceed until the concentration of compounds un-able to pass becomes equal on both sides of the membrane. The postulated increase in ion permeability of the membrane can be expected to decrease the overall tissue impedance. It has been shown, however, that the decrease of the membrane resist-ance of a relatively low figure has only a small effect on the tissue impedance. This effect may be masked completely by the ef-fect of the loss of ions from the intercellu-lar compartment (Van Harreveld and Ochs, '56).

The postulated membrane changes can not only explain the impedance increase after circulatory arrest and during spread-ing depression, but also other features like the slow potential changes and the de-pression of the electrocorticogram during spreading depression (Van Harreveld and Ochs, '56). The demonstration of a swell-ing of the nerve cells and apical dendrite after circulatory arrest (Van Harreveld, '57) and of apical dendrites during spread-ing depression (Van Harreveld, '58) sup-ported the concept developed above. It is further strengthened by the transport of chloride ions described in the present paper.

It has been suggested recently that as-phyxial potentials and the slow potential change accompanying spreading depres-sion are generated at the blood-brain bar-rier (Tsirgi and Taylor, '58). Some of the features of these potentials described in the literature are hard to reconcile with this view. The present investigation shows that such potentials are the concomitants of major ionic movements in neuronal structures which can account for the po-tentials recorded.

SUMMARY

A histochemical method for the local-ization of chloride ions (and possibly other anions) in tissues was described which was used to visualize the position of these ions in the cerebral cortex under various experimental conditions. A comparison was made between the chloride distribu-tion in normal oxygenated cortex of rats, rabbits and cats, and in cortex in which the rapid impedance increase had taken place which develops a few minutes after circulatory arrest. In the control cortices the chloride was rather evenly distributed. In preparations of cortex in which the asphyxial impedance change had taken

place the amount of chloride in many of the apical dendrites had increased markedly. In other apical dendrites and in the perikarya a smaller increase of the chloride content was noted. The tissue in between the apical dendrites appeared to have lost chloride.

During spreading depression a similar transport of chloride into apical dendrites occurred. It was less pronounced than the transport observed after circulatory arrest. Spreading depression does not normally invade the retrosplenial area. It was found that the chloride transport into the apical dendrites stops rather sharply at the parasagittal sulcus which forms the lateral boundary of this area.

On the basis of cortical impedance changes it was postulated previously that after cortical asphyxiation and during spreading depression sodium chloride moves from intercellular spaces into neuronal elements of the cortex. This postulate is greatly strengthened by the observed chloride movements.

ADDENDUM

Electronmicroscopy[2] of cortices frozen in the way described above supports the postulate that the peripheral position of the chloride in the apical dendrites is an artefact, caused by the formation of an ice core in these structures. The electronmicrograph shown in figure 11B is of cortex which, after the initial freezing with isopentane, was kept for a week at −25°C in alcohol without silver nitrate. There are many empty areas surrounded by material which has a moderate electron density. It was very difficult to recognize in such preparations any but the most gross structures, like perikarya and apical dendrites. This picture is consistent with the concept that the granular appearance of these preparations seen with the light microscope is due to the formation of ice crystals. The latter would be represented by the clear spaces whereas the electron dense material would represent the protoplasma which, with all its solutes, had been displaced and concentrated by the developing ice crystals.

Figure 11A shows an electronmicrograph of asphyxiated cortex treated in the same way with the exception that alcohol saturated with silver nitrate was used for the substitution fixation. These preparations showed fine black dots of high electron density which represent the, perhaps partly reduced, silver chloride crystals. It was possible to recognize in these preparations apical dendrites as shown in figure 11A. The center of this structure is clear which indicates that in the frozen state it was occupied by a central ice core. The black, silver containing granules are situated partly in and partly against the protoplasma pushed by the ice core to the periphery of the apical dendrite. Little chloride was present in the tissue surrounding the dendrite.

Electronmicrographs of cortices impregnated according to Cajal's method B had an entirely different appearance (fig. 11C). These preparations contained numerous, much larger and less well defined granules which may represent the protein-silver compound formed with this method. The darker structures may represent fibers. These preparations support the view that the histochemical method for chloride described above is essentially different from the usual histological silver impregnation methods.

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[2] We are indebted to Dr. B. Henke, Professor of Physics at Pomona College for the use of an electronmicroscope and to Mr. M. Mendelson for the preparation of the material for electronmicroscopy.

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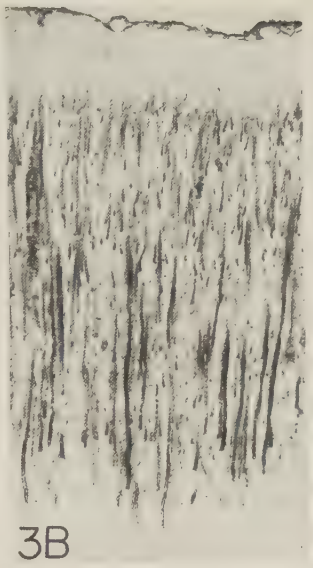
PLATE 1

EXPLANATION OF FIGURES

3A, B and C Photomicrographs of rat cortex. The chloride distribution is shown in A and B. A is a section of cortex frozen while the circulation was intact, B of cortex after the asphyxial impedance change had taken place. A green filter was used in making all photomicrographs used in this paper. This filter made the yellow-brown subhalide appear darker on the photographs than it was in the sections thus emphasizing the differences between control and experimental preparations. C is a section of an asphyxiated rat cortex treated like A and B but in addition stained with gallocyanin. The 5 upper cortical layers are marked. The calibration line indicates 250 μ.

4A, B and C Photomicrographs of rabbit cortex. The chloride distribution while the circulation is intact is shown in A, that after the asphyxial impedance change in B. C is a section of cortex frozen before the rapid impedance change had taken place stained with gallocyanin. The 4 upper cortical layers are marked. The calibration line indicates 250 μ.


3A


3B


I
II
III
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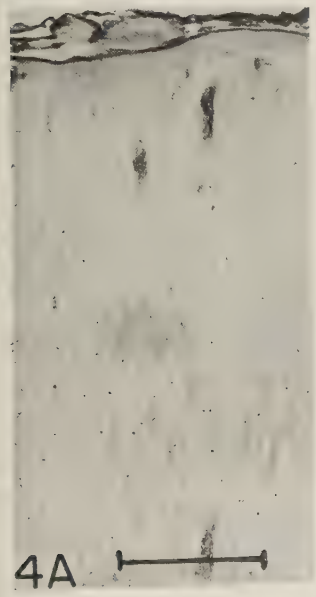
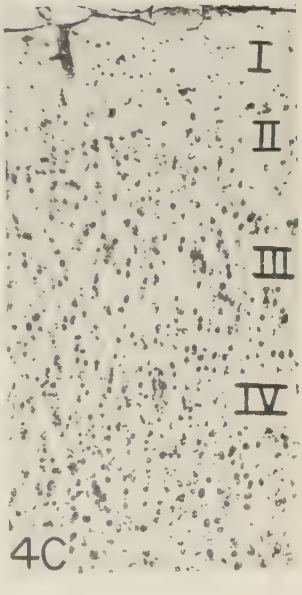
3C


4A


4B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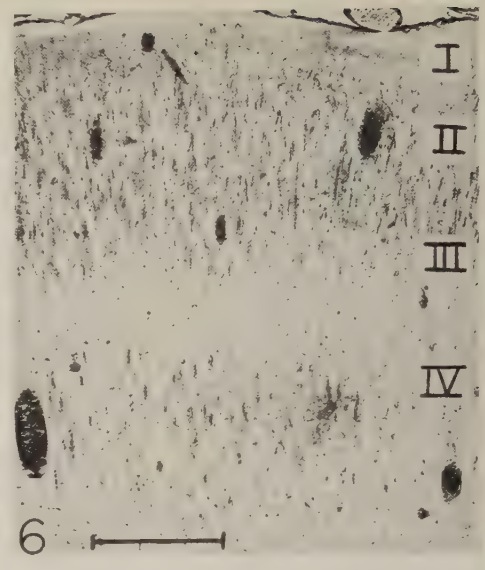
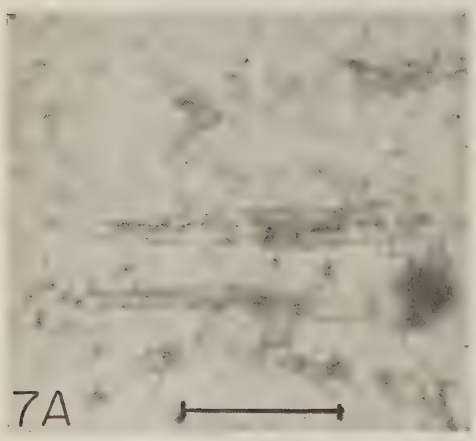
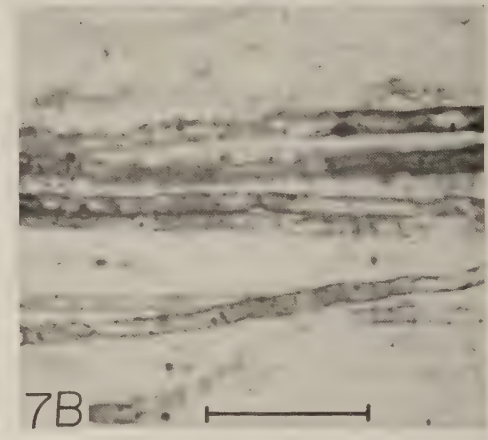


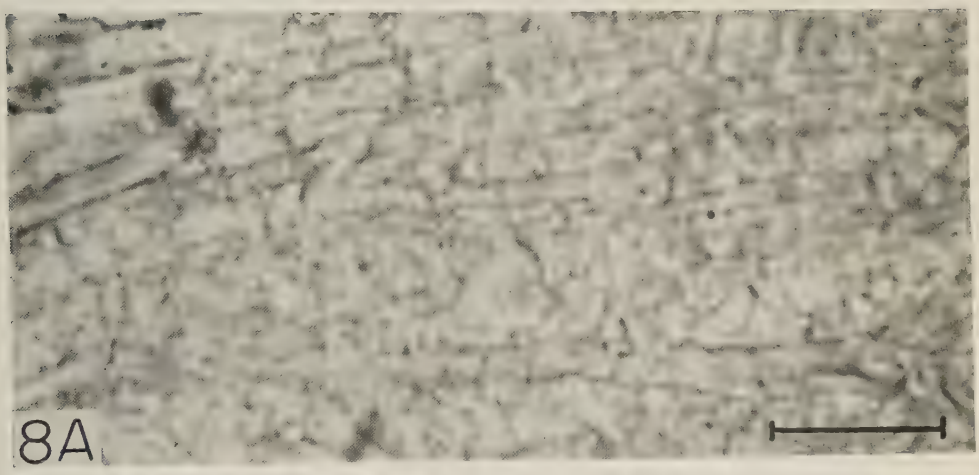


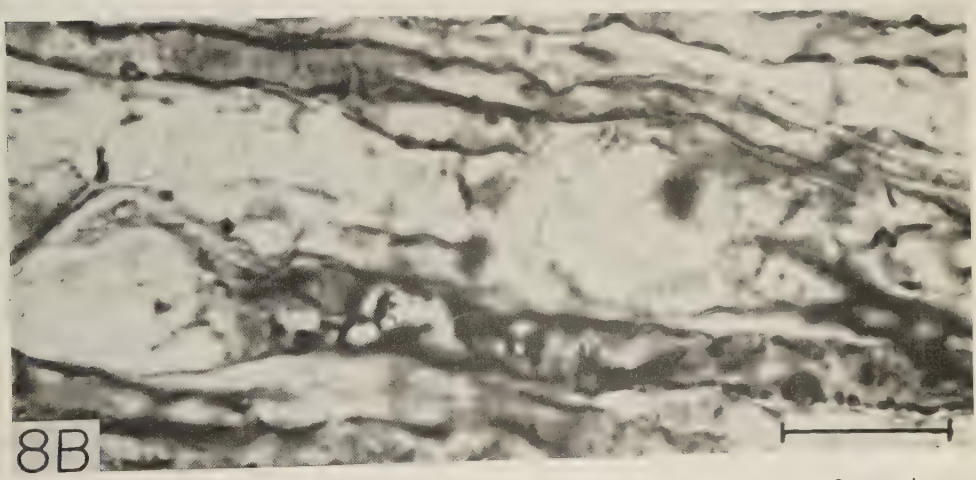


5 A cross section of the m. rectus abdominus of the rat. The chloride is mainly located outside the muscle fibers and in the connective tissue of the fascia. The calibration line indicates 250 μ.

6 Demonstrates the chloride distribution in a section of rabbit cortex at the height of a spreading depression. The position of the upper 4 cortical layers is indicated. The calibration line indicates 250 μ.

7A and B Sections of rat cortex at higher magnification. Cortex A was frozen while the circulation was intact, B after the asphyxial impedance rise had taken place. The sections show the chloride distribution. Some apical dendrites in the asphyxiated cortex are quite dark, others remained light. The tissue in between the apical dendrites is considerably lighter in the cortex frozen after the impedance rise. Notice the difference in diameter of the apical dendrites under the two experimental conditions. The calibration line indicates 25 μ.





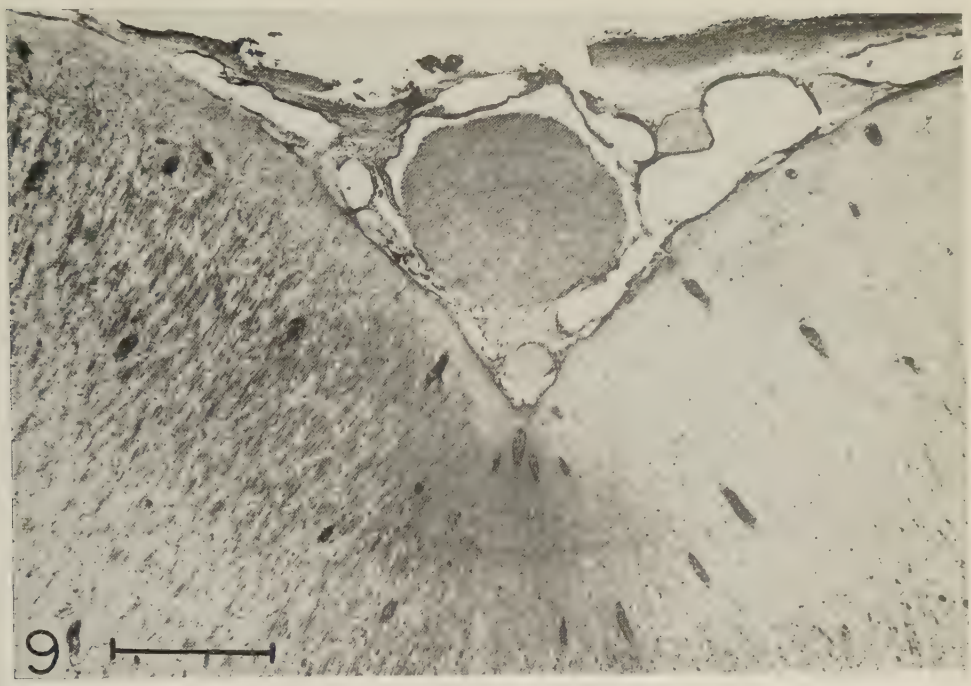
8A and B Sections of rabbit cortex at high magnification (oil immersion). Cortex A was frozen while the circulation was intact, B after the asphyxial impedance increase. The dark boundary lines of the apical dendrites and the foam-like appearance of the tissue in between them is well demonstrated in the control cortex (A). B shows the distribution of chloride in apical dendrites after the asphyxial impedance increase. The calibration line indicates 10 μ.

PLATE 4

EXPLANATION OF FIGURES

9 A section of rabbit cortex on both sides of the parasagittal sulcus. The cortex was frozen at the height of a spreading depression. The retrosplenial area is to the right of the sulcus. The darkening of the apical dendrites stops at the sulcus. The green filter which was used to enhance the contrast also made the retrosplenial area too light as compared with the cortex to the left of the parasagittal sulcus. In reality the two parts of the cortex were of the same shade of brown. The calibration line indicates 250 μ.

10 The same cortical region as in figure 9. It was taken from a cortex frozen after the asphyxial impedance change had taken place. The retrosplenial area is to the right of the parasagittal sulcus. The darkening of apical dendrites typical for cortex frozen after the impedance rise is present on both sides of the parasagittal sulcus. A number of blood vessels were found in the dark spots immediately under the sulcus. The calibration line indicates 250 μ.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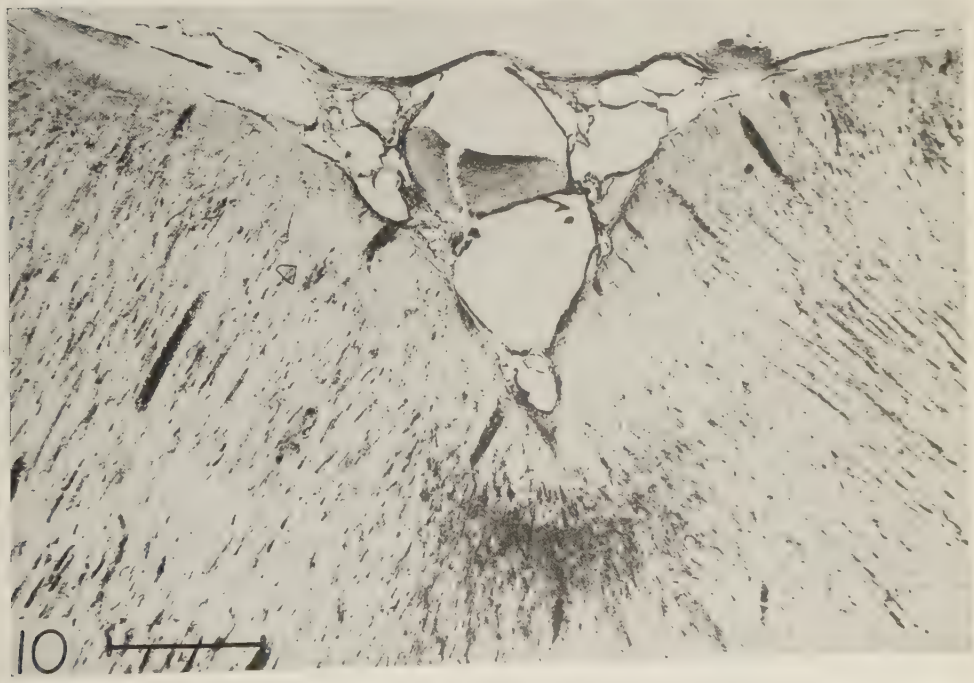
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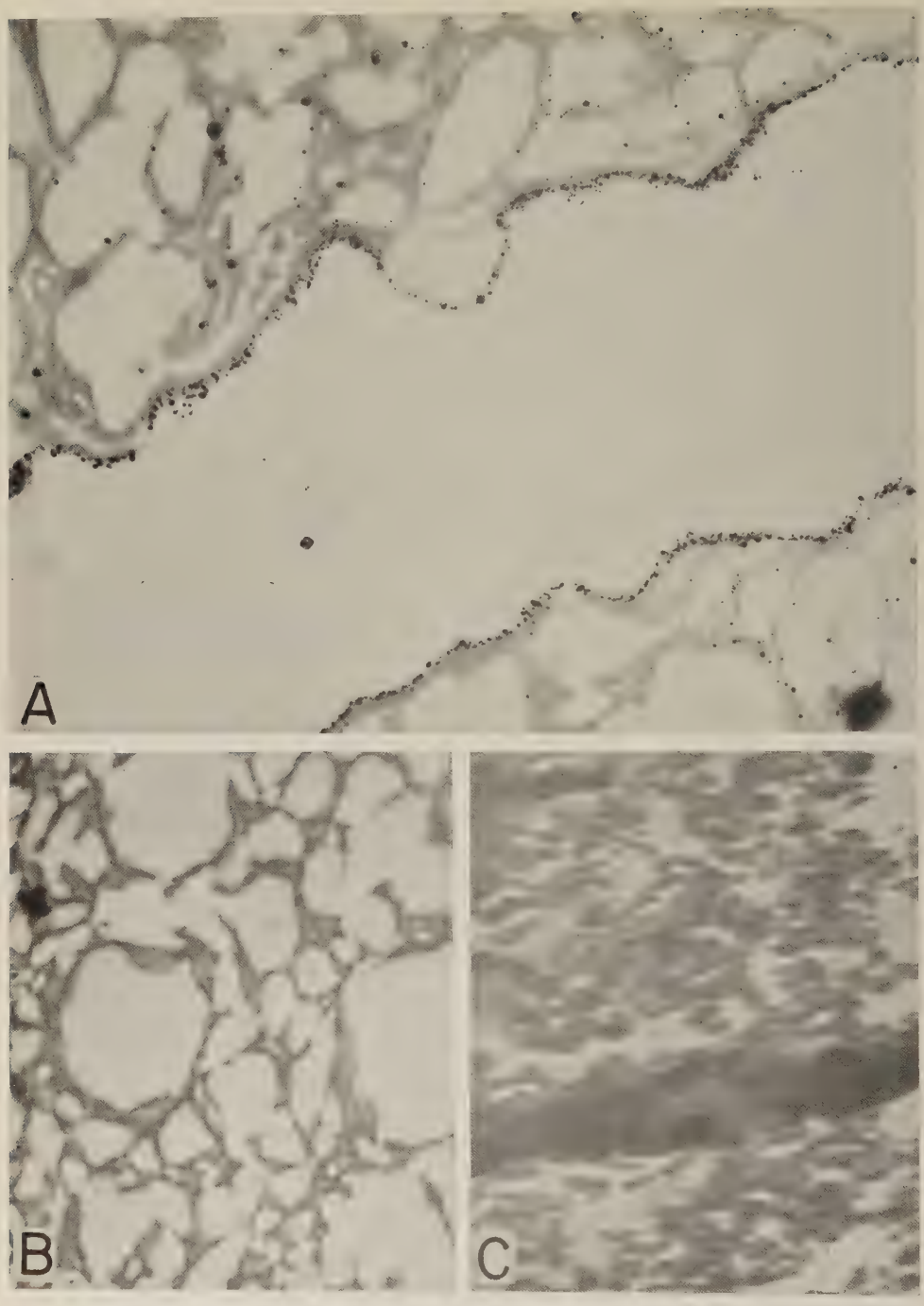

11 A is an electronmicrograph of asphyxiated cortex treated with the histo-chemical method for chloride. The large structure which runs from the bottom of the picture on the left to the top on the right is an apical dendrite. The black material is silver chloride. B is of cortex treated in the same way with the exception that the alcohol for the substitution fixation did not contain silver nitrate. The clear areas in this picture and in A represent ice crystals. C is an electronmicrograph of cortex treated with Cajal's method B. × 16,000.

Glutamate-Induced Contractions in Crustacean Muscle[1]

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L-glutamate elicits muscle contractions n crustaceans (Robbins, '58; Van Harreeld, '59). The mean threshold concentraon for this effect in *Cambarus clarkii* was .035 mM/1 or 5 γ/ml. Several compounds elated to glutamic acid either chemically r metabolically were inactive or caused ontractions at much higher concentraons. The most active of these compounds vas L-glutamine which was about 70 times ess active than L-glutamate. Still less ac-ve were L-aspartate and D-glutamate. Other compounds tested (L-α-amino adipic cid, L-asparagine, glutaric acid, succinic cid, L-ornithine, L-proline, DL-α-amino utyric acid, γ-amino butyric acid, α-keto lutaric acid) did not cause contractions in oncentrations up to 20 mM/l. The ability f glutamic acid to cause muscle contracions in *Cambarus* thus seems to be quite pecific.

In the present investigation the above bservations were extended to some marine rustaceans. Furthermore the mechanal and electrical features of the conractions induced by glutamate in the adductor and abductor muscles of the *Cambarus* claw were investigated.

METHODS

The methods of mechanical and electrical recording were all conventional. Contractions of the adductor muscle were recorded on smoked drums with isometric, those of the abductor muscle with isotonic levers. Induction coils were used for stimulation.

The electrodes for extracellular recording of action potentials consisted of silver-chloride plated silver wires. For intracellular recording micropipettes filled with 3 M potassium chloride were used. The potentials were led into a cathode follower directly coupled with a type 333 Dumont oscilloscope. This instrument had sufficient built-in amplification to make preamplification unnecessary. The glutamate solutions used had a pH of 7.

RESULTS

Muscle contractions caused by L-glutamate in marine crustaceans. In three species of marine crustaceans (*Panulirus interruptus*—spiny lobster, *Cancer antennarius*—edible crab, and *Pachygrapsus crassipes*—shore crab) the application of L-glutamate to muscles caused contractions as in *Cambarus*. The threshold concentration was determined as described previously (Van Harreveld, '59). When investigating a claw muscle, a short piece of rubber tubing was fitted on the fixed member of the pincer after its tip had been cut off. The tube was closed with a clamp. A hypodermic needle was introduced through the wall of the tube into the propodite. Neutralized L-glutamic acid dissolved in seawater was injected through the needle into the claw. The fluid ran out through the ischiopodite. By injecting seawater through the needle the glutamate solution was washed out again. When using walking legs it was found more convenient to fit the rubber tube on the meropodite and perfuse the leg in the opposite direction. In determining threshold concentrations a careful study of the movements caused by the injection is necessary to distinguish active contractions from passive movements caused by pressure changes in the claw or leg.

The threshold was determined in the walking legs of three specimens of *Panulirus*. Considerable individual differences in threshold were found. In two specimens the mean threshold was 0.05 mM/l in a third it was 0.3 mM/l. The variation

[1] This investigation was supported in part by a research grant from the National Institute of Neurological Diseases and Blindness (B-340).

in threshold of the legs of one individual was small. For instance 9 legs of one of the lobsters were examined. The mean threshold was 0.05 mM/l with a maximum of 0.075 mM/l and a minimum of 0.038 mM/l. The mean threshold of 14 claws of Pachygrapsus was 0.12 mM/l with a standard error of 0.01 mM/l. The walking legs of three specimens of *Cancer* had mean thresholds of 0.17, 0.12 and 0.14 mM/l.

The glutamate-induced contraction. Figure 1 shows an isometric contraction of the adductor muscle of *Cambarus* produced by the rapid injection of 1 ml of 0.01 M L-glutamate in physiological salt solution (Van Harreveld, '36) through the fixed member into the claw. The contraction was quite strong and was of short duration even though the glutamate remained in the muscle. After perfusing with physiological solution for several minutes to wash away the amino acid, the muscle could be made to contract again with glutamate. Similar contractions were obtained by the application of this compound to the abductor muscle.

The glutamate induced contractions were accompanied by potential changes. The potential shown in figure 2a was led off from two silver-chloride plated silver wires, one of which was placed in the tubing fitted on the fixed member of the pincer. The other was stuck into the adductor muscle through a hole in the exoskeleton of the propodite. This hole was on the side of the fixed member about at the middle of the muscle. The potentials produced in this way were in general quite complicated and were large (up to about 8 mv) considerably larger than the action potentials caused by indirect stimulation. After perfusing the claw with physiological solution potential changes could again b elicited by glutamate. The potentials va ied a great deal in shape from preparatio to preparation, and in the same claw wi repetition of the injection.

Intracellular electrodes were used in number of experiments to record potenti changes from the adductor muscle. M cropipettes were lowered onto the musc surface which had been exposed by r moving the exoskeleton over the abducto muscle and pulling the latter out by i tendon. Penetration of a fiber caused th characteristic jump in potential which i the best preparations was of the order o 70 mv. To minimize mechanical distu bances the pincer was fixed in maxim opening. Furthermore the penetration wa made close to the origin of the fiber on th exoskeleton. Glutamate was applie through the fixed member of the claw.

The records obtained varied greatl Some were very irregular which may hav been caused by mechanical disturbance due to the contraction. In those in whic there were no obvious mechanical effec the variability may have been due to th uncontrolled speed of application and th varying pattern of distribution of the glu tamate on the muscle fiber. The recor shown in figure 2b was chosen because o its simplicity which may indicate the ab sence of mechanical effects and a speed and uniform application of glutamate t the surface of the muscle fiber. The ap plication of the amino acid caused d polarization which rapidly reached a max imum and then declined in the course o about 0.2 sec. This initial depolarizatio was in some experiments of considerabl longer duration and often showed severa maxima. Also it sometimes showed notch es as in figure 2b. The magnitude varie

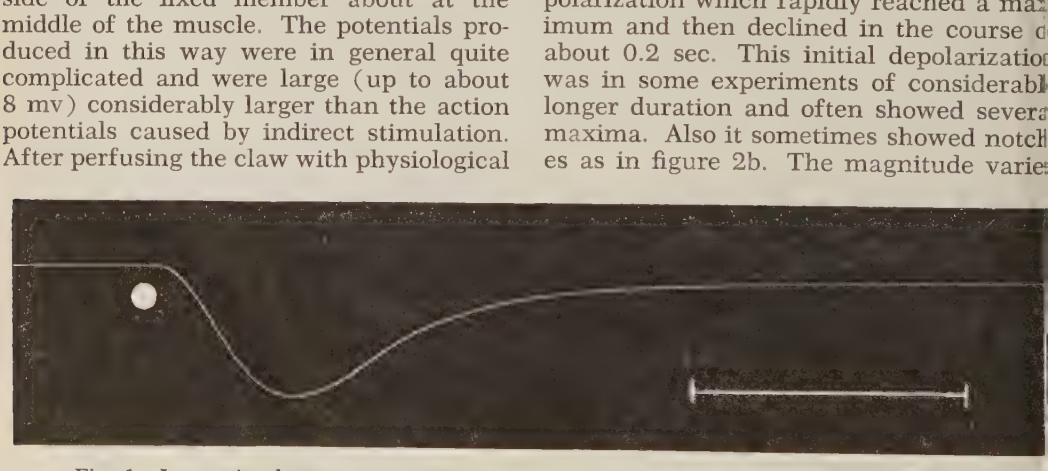

Fig. 1 Isometric glutamate contraction. At the dot 1 cm^3 0.01 M glutamate in physiological solution is injected. The horizontal line indicates 1 sec.

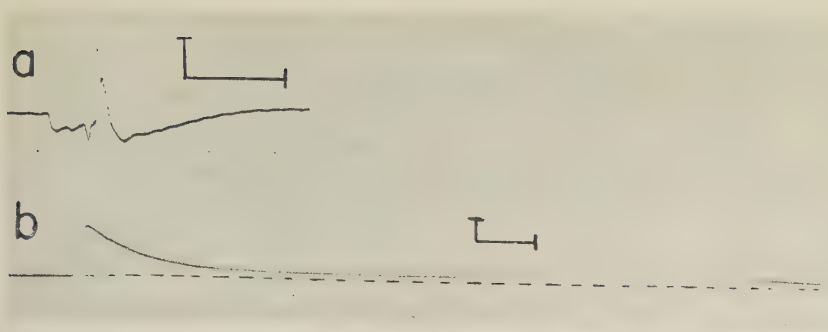

Fig. 2 a, Electrical response of the adductor muscle to the injection of 1 cm^3 0.01 M glutamate and recorded with extracellular electrodes. The record shows an AC ripple. The horizontal line indicates 0.1 sec., the vertical 5 mv. b, Potential change induced by the application of glutamate 0.01 M and recorded from the adductor muscle with an intracellular electrode. Horizontal line indicates 0.1 sec., the vertical 10 mv.

between 20 and 35 mv. There remained a residual depolarization a few millivolts in magnitude which decreased slowly in 5 to 10 sec.

After-effects of glutamate application. After administration of glutamate the adductor muscle responded less or not at all to indirect stimulation. The adductor muscle of *Cambarus* is innervated by three nerve fibers, one of which is an inhibitory one, the other two are motor axons. Of the latter, one causes a contraction when stimulated with single shocks, and a fast and strong contraction when stimulated faradically. The other axon elicits muscle contractions only when stimulated faradically. These contractions develop slowly and are weaker when the stimulation is not prolonged for a considerable period (Van Harreveld and Wiersma, '36). The two motor fibers were isolated in a number of claws. The "fast" axon was stimulated faradically at a frequency of about 30/sec. for 0.25 sec., using a mechanically driven switch which energized an induction coil. The "slow" axon was stimulated for periods of 2 sec. at the same frequency. Physiological solution (1 ml) containing various concentrations of glutamate was administered to the adductor muscle through the fixed member of the claw. Shortly after the resulting contraction had subsided the nerve fibers were stimulated. Then the preparation was perfused for a few minutes with physiological salt solution, after which the axons were again stimulated, etc.

Figure 3 shows an experiment in which the fast axon was stimulated. Record 3a shows the contraction before the administration of glutamate (0.025 M), which caused the large contraction in record b. Stimulation of the fast axon shortly afterward elicited only the small contraction of record c. Records d, e, f and g were taken at intervals of a few minutes during which the claw was perfused with physiological solution. They show the slow return of the contraction on indirect stimulation. Even though a high concentration of glutamate was used the contraction was not depressed completely. This may be due to an incomplete suppression of the contraction in individual muscle fibers. It is also possible, however, that the amino acid did not reach all the muscle fibers.

Figure 4 is from an experiment in which the slow axon was stimulated. Record 4a shows two stimulations before the administration of glutamate (0.005 M). After the glutamate contraction (at the dot) had subsided, stimulation had hardly any effect (b). Record c was made after a few minutes of perfusion with physiological solution; d, after additional perfusion. The slow contractions were suppressed by lower concentrations of glutamate than the fast ones. The lowest concentration which caused a decrease of the slow contraction was 0.0005 M, about 10 times the threshold concentration for contraction. The depressing effect of glutamate is quite reversible even when high concentrations (0.025 M) are used.

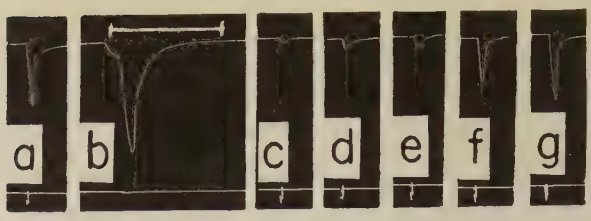

Fig. 3 Effect of glutamate on the fast contraction. Upper trace shows isometric contractions of the adductor muscle caused by stimulation of the "fast" fiber. Lower trace indicates short (0.25/sec.) faradic stimulations. Record a shows a control contraction, b, injection of 1 cm^3 0.025 M glutamate. c, Effect of stimulation shortly afterwards, d, after perfusion of the muscle for a few minutes with physiological solution. Each of records e, f and g was taken after a few additional minutes of perfusion. The horizontal line in b indicates 10 sec.

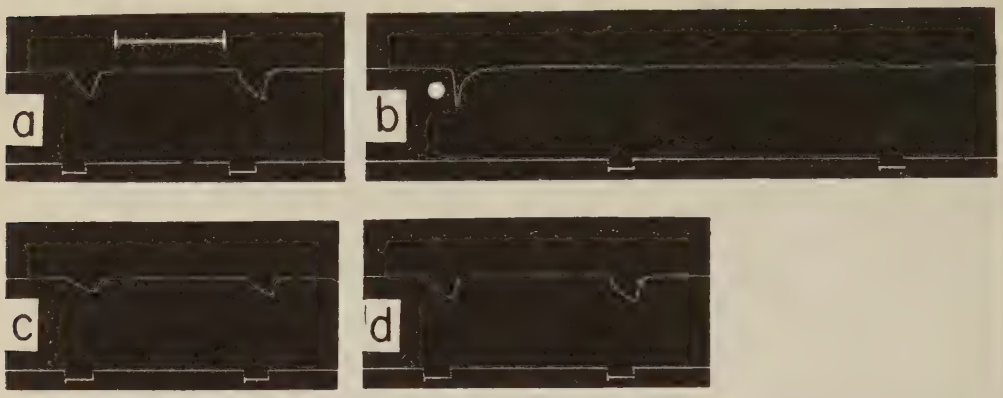

Fig. 4 Effect of glutamate on the slow contraction. Upper trace shows isometric contractions of the adductor muscle caused by stimulation of the "slow" fiber. Lower trace indicates the duration of the faradic stimulation (2 sec.). Record a shows two control contractions. Due to latent facilitation the second contraction develops more rapidly and becomes larger than the first. In b, 1 cm^3 0.005 M glutamate was injected at the dot. This was followed by two stimulations which caused just visible contractions. The muscle was then perfused with physiological solution for a few minutes and record c was made. After additional perfusion stimulation of the slow fiber caused contractions almost as large as before the glutamate injection (d). The horizontal line indicates 10 sec.

The after-effect of a glutamate application might vanish even without washing. To examine this possibility the contraction was slightly depressed by injection of the amino acid at low concentration. Then the contraction height was determined at regular intervals without perfusing the muscle. Only a slight increase in height was observed over a period of 30 to 45 min. This shows that the removal of the glutamate by perfusion is a material factor in the reversibility of the depression.

Although glutamate suppressed the contractions caused by stimulation of the motor nerve fibers, it did not prevent contractions elicited by direct faradic muscle stimulation, as shown in figure 5.

The effect of glutamate on the muscle action potentials elicited by indirect stimulation was investigated. The electrode arrangement for extracellular recording of action potentials described above was used. Figure 6a shows the potentials elicited by stimulating the fast axon with a single shock. The injection of 1 cm^3 0.01 M glutamate reduced its size greatly (b). After one minute the potential was still smaller (c). However, after perfusing the muscle for a few minutes with physiological solution it had gained considerably (d). Additional perfusions made the potential still larger (e and f). It became even larger than before the injection. Since the shape of this action potential differs from the

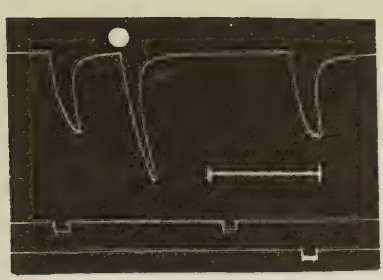

Fig. 5 Effect of glutamate on isometric contractions of the adductor muscle elicited by direct and indirect stimulation. Upper trace shows isometric contractions. The upper signal trace indicates stimulation of the nerve bundle in the meropodite, the lower trace direct stimulation of the adductor muscle. At the dot injection of 1 cm³ 0.025 M glutamate into the claw. This abolishes the effect of indirect but not of direct stimulation of the muscle. The horizontal line indicates 10 sec.

preglutamate potential, no great significance should perhaps be attached to this fact.

Figure 7 shows a similar experiment in which the slow axon was stimulated at a frequency of about 17/sec. A marked facilitation of the action potential was present during the stimulation which lasted 2–2.5 sec. Record 7a was taken before glutamate administration. After injection of 1 ml 0.005 M of this amino acid record b shows the absence of action potentials. However, perfusing the muscle for a few minutes brought them back (c). They grew larger after additional perfusion (d, e). Again the action potential after recovery was larger than during the control stimulation.

Brockman and Burson ('57) described the after-effect of glutamate administra-

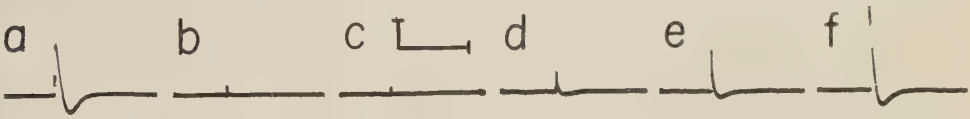

Fig. 6 Effect of glutamate on the action potential of the adductor muscle caused by stimulation of the "fast" fiber with single shocks. Record a shows the action potential before administration of glutamate. After injection of 1 cm³ 0.01 M glutamate b was taken and one minute later c was recorded, then the muscle was perfused for a few minutes with physiological solution and d was made. After additional perfusion e and f were recorded. The horizontal line indicates 0.2 sec., the vertical 100 μv.

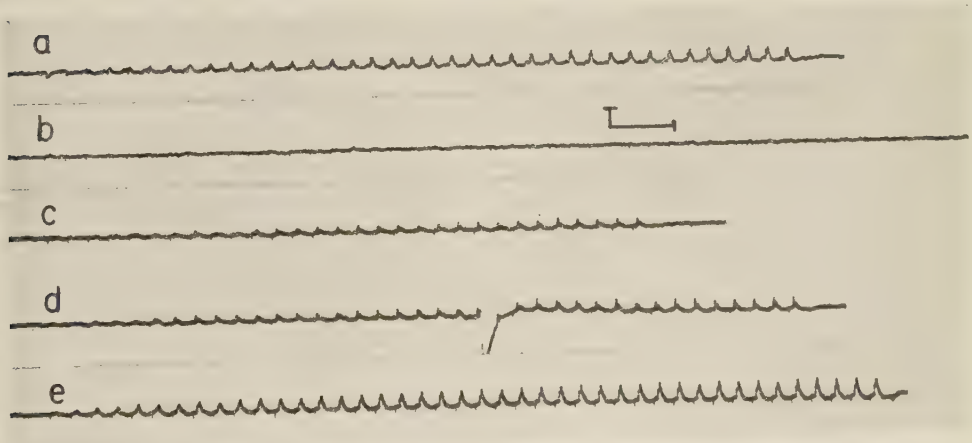

Fig. 7 Effect of glutamate on the action potentials of the adductor muscle caused by stimulation of the "slow" fiber at a frequency of about 17/sec. Record a shows the action potentials before glutamate administration. Then 1 cm³ 0.005 M glutamate was injected and b was taken directly afterwards. Record c was made after a few minutes perfusion of the muscle with physiological solution, d and e after additional perfusion. The horizontal line indicates 0.2 sec., the vertical line 10 μv.

tion on the mechanical effect of indirect stimulation, but did not mention the contraction which is caused by the application of this amino acid.

Effect of glutamate on crustacean nerve. The effect of L-glutamate on crustacean nerve was examined by bathing nerve bundles dissected from the meropodite for periods up to 30 minutes in glutamate solutions. The highest concentration used was 0.025 M in physiological solution. Action potentials were led off from the nerve before and after this treatment. As mentioned previously (Van Harreveld, '59) no appreciable effect on the action potentials was observed.

Inhibition of contractions induced by glutamate. Peripheral inhibition of striated muscle is a feature of crustaceans. The abductor muscle of the claw is the classical preparation in which Biedermann (1888) found the first clear evidence for this type of inhibition. The muscle is innervated by two nerve fibers only, a motor fiber and an inhibitory one. Hoffmann ('14) showed that these fibers run in different nerve bundles in the meropodite.

The preparation used in the present investigation consisted of a *Cambarus* claw from which most of the exoskeleton covering the adductor muscle had been removed. This muscle was then torn out leaving the abductor muscle exposed. In order to produce contractions of the muscle, glutamate had to be applied more or less simultaneously to a considerable portion of the muscle fibers. Just dripping glutamate solutions on the surface did not seem to satisfy this condition since the contractions were small and varying in magnitude. Better results were obtained by forcing the glutamate solution under a constant pressure through a capillary tube directed toward the surface of the muscle. The amino acid was washed out again by forcing physiological solution through the same tube. A series of reasonably uniform contractions were obtained by applying alternately the glutamate jet for 1.5 sec. and washing for three minutes.

No attempt was made to isolate the inhibitory axons. Instead the nerve bundle in the meropodite which contains the fiber was stimulated. In this bundle are also present the excitatory axons for the adductor muscle, but since this muscle had been removed no interference with the abductor contractions was to be expected. Indeed stimulation of this bundle alone had no visible effect on the muscle record. The motor axon for the abductor muscle runs in another nerve bundle in the meropodite. Figure 8a shows the effect of faradic stimulation of the two bundles. The bundle containing the motor axon was stimulated for about 4 sec. The inhibitory bundle was stimulated for some time during this period causing a complete relaxation of the muscle. At the moment the inhibitory stimulation was stopped the muscle resumed its contraction, to relax again at the end of the motor stimulation.

The effects of inhibition on glutamate induced contractions in the same preparation is shown in figures 8b–f. Records 8b, d and f are controls to show that satisfactorily uniform contractions result from the application of the glutamate jet. In record 8c the inhibitory bundle was stimulated after the glutamate contraction had started. This caused relaxation of the muscle, greatly curtailing the contraction as compared with the control contractions (8b and d). It is of interest to note that contrary to the inhibition of contractions induced by nerve stimulation the glutamate contraction is not resumed at the end of the inhibitory stimulus. In record e inhibition was started before the application of glutamate, which had hardly any effect under these circumstances. From control experiments in which jets of physiological solution were applied it is known that small effects as shown in this record are often artifacts due to the mechanical effect of the jet of fluid hitting the muscle surface. This record thus shows that the glutamate contraction can greatly and perhaps completely be inhibited by stimulation of the inhibitory nerve fiber for the abductor muscle.

Antagonism between glutamate and γ-amino butyrate. McLennan ('57) and also Brockman and Burson ('57) have shown that γ-amino butyric acid in relatively high concentrations (40–100 γ/ml.) depresses contractions of the adductor and abductor muscles of the crayfish claw. In view of the possible function of γ-amino butyric acid as an inhibitory transmitter substance

crustacean muscle (Boistel and Fatt, 3) it was of interest to investigate the fect of this amino acid on glutamate in-ced contractions.

Glutamate was dissolved in physio-gical solution to a concentration of 0.01 . A second solution was prepared with L-glutamate and γ-amino butyrate both in a concentration of 0.01 M; and a third solution with 0.01 M of L-glutamate and L-aspartate. Dilutions were made of these solutions and the threshold concentrations for contraction of the adductor muscles of *Cambarus* were determined as described

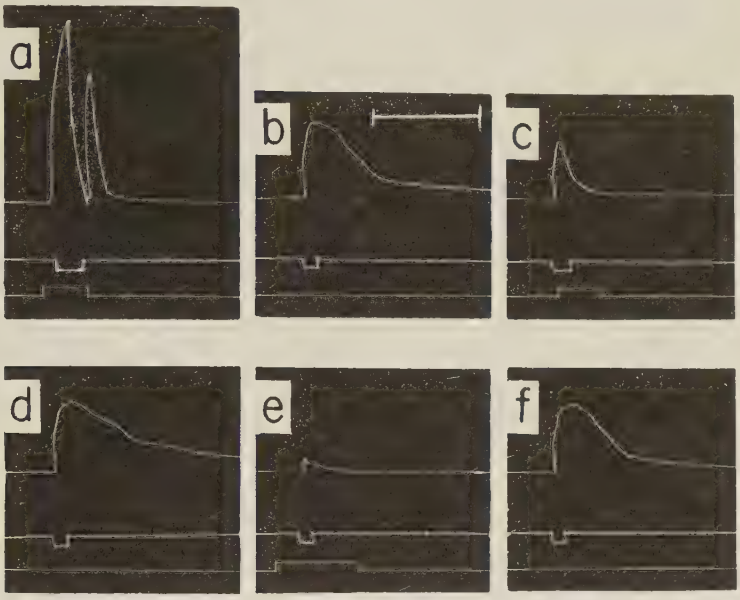

Fig. 8 Effect of inhibition on nervous and glutamate induced contractions. Record a shows an isotonic muscle contraction elicited by faradic stimulation of the motor fiber for the abductor muscle, which is inhibited by stimulation of the inhibitor axon. The inhibitory stimulus is indicated by the upper, the excitatory stimulation by the lower signal trace. In b, c, d, e and f short jets of a 0.01 M glutamate solution are applied. The opening of the valve controlling the jet is indicated by the upper signal trace. In c and e the resulting contractions are inhibited. The inhibitory stimulus is indicated by the lower signal trace. In c the inhibitory stimulus is started during the glutamate application, in e before. The horizontal line in b indicates 10 sec.

TABLE 1

Threshold concentrations of L-glutamate in the presence of equimolar concentrations of L-aspartate and γ-amino butyrate (millimoles per liter)

No.	L-glutamate	L-glutamate + L-aspartate	L-glutamate + γ-amino butyrate
	mM/l	*mM/l*	*mM/l*
1	0.038	0.038	0.15
2	0.025	0.038	0.15
3	0.038	0.038	0.20
4	0.038	0.038	0.15
5	0.050	0.050	0.20
6	0.038	0.038	0.15
7	0.038	0.025	0.20
8	0.038	0.025	0.08
9	0.038	0.038	0.15
10	0.038	0.050	0.30
Means	0.038 ± 0.002	0.038 ± 0.002	0.17 ± 0.02

above. The results of 10 experiments are shown in table 1. The threshold for glutamate alone was between 0.025 and 0.05 mM/l, the mean being 0.038 mM/l. The threshold concentration was the same for the solution containing both glutamate and aspartate, showing that the presence of a second amino acid was without effect on the glutamate contraction. The threshold for the solution containing an equimolar amount of glutamate and γ-amino butyrate was higher, varying between 0.08 and 0.30 mM/l with a mean of 0.17 mM/l. This is statistically a highly significant difference ($P < 0.01$) showing that γ-amino butyric acid antagonizes the glutamate induced contraction.

DISCUSSION

L-glutamate in relatively low concentrations caused muscle contractions in one fresh water crustacean (*Cambarus clarkii*) and in three species of marine crustaceans (*Panulirus interruptus, Cancer antennarius* and *Pachygrapsus crassipes*). This effect could be elicited many times when the glutamate was washed away between applications, even when high concentrations of the amino acid were used. This compound thus does not seem to have a deleterious effect on the muscle. Glutamate had no appreciable effect on crustacean nerve which suggests that the contraction is due to an effect on the myo-neural junction or on the muscle itself. After the application of glutamate the contractions as well as the action potentials of the adductor muscle caused by stimulation of the "fast" and the "slow" fiber were greatly reduced. This suggests that glutamate acts on the myo-neural junction since a large component of the action potential is a "pure" junctional potential caused by the release of a transmitter substance which increases the conductivity of the muscle membrane at the junction (Hoyle and Wiersma, '58a). The observation that direct muscle stimulation remains effective in glutamate-treated muscles supports this view. The stimulating action of glutamate on the muscle and the depressing effect on contractions caused by indirect stimulation are comparable with the action of acetylcholine on vertebrate muscles (Brown, Dale and Feldberg, '36, Brown, '37). It may be justified to draw the parallel between the action of glutamate and acetylcholine further by assuming that glutamate causes depolarization of a specialized part of the muscle membrane at the crustacean myoneural junction just as acetylcholine depolarizes the vertebrate motor endplate (Kuffler, '43, Fatt, '50). Such a depolarization may start the chain of events leading to muscle contraction like the depolarization at the myo-neural junction produced by an incoming nerve impulse. The glutamate remaining in the muscle may make the muscle membrane at the myoneural junction less responsive, which could be due to a maintained depolarization of the membrane, or to its "desensitization." The latter was observed in the endplates of frog muscle by Katz and Thesleff ('57), who found that during and after a sustained application of acetylcholine the responsiveness of the endplate to short test applications of this compound is decreased. Since the residual depolarization is quite small, it seems likely that desensitization rather than depolarization is the cause of the glutamate effect on indirect stimulation.

Hoyle and Wiersma ('58a) analyzed crustacean muscle action potentials recorded with intracellular electrodes and elicited by indirect stimulation. The "pure" junctional potentials mentioned above were found to be complicated by "abortive" activity in the surrounding membrane (local response) and by spike potentials. The latter were relatively difficult to elicit (see also Fatt and Ginsborg, '58). Finally there would be a very slow response due to "lingering" transmitter substance. The junctional potentials in the adductor muscle of *Cambarus* varied greatly in magnitude. Junctional potentials as large as 20 mv have been recorded as a result of stimulation of the fast axon in this species.

The potentials led off with intracellular electrodes during glutamate administration seem to be consistent with the postulate that glutamate causes depolarization at the myo-neural junction. The initial depolarization may have the value of a junctional potential, consisting of a direct effect of glutamate on the muscle mem-

rane at the myo-neural junction, perhaps mplicated by local responses in the surounding membrane. The long duration nd large size of this effect as compared ith the junctional potentials caused by ervous stimulation may be due to the ersistent presence of glutamate. Its deine may be due to "desensitization" of ne membrane. Indications of spike acvity were observed in only a few experiments. The notched appearance of the rescente of the potential shown in figure b may be an indication of this. The mall residual depolarization may be the xpression of a long lasting effect of gluamate on the specialized membrane at ne junction.

McLennan ('57) observed inhibition of ne fast as well as of the slow contraction f the adductor muscle by γ-amino butyric cid in concentrations of 40 to 100 γ/ml. lso Brockman and Burson ('57) found hat contractions of the abductor muscle nd the slow contraction of the adductor an be depressed by γ-amino butyric acid. n the experience of Wiersma and Hoyle '58), however, this amino acid does not nimic the effect of inhibitory stimulation ither mechanically or in its effect on the nembrane potential. Boistel and Fatt '58), on the other hand found that γamino butyric acid increases the chloride ermeability of the membrane as does inibitory stimulation. It was found in the resent experiments that the threshold for he glutamate induced contractions is markedly increased by the simultaneous dministration of an equimolar amount of -amino butyrate. There seems to exist an ntagonism between the action of these amino acids as far as their effect on the muscle is concerned.

The contractions of the abductor muscle elicited by the application of glutamate could be inhibited by nervous stimulation. Inhibition in crustacean muscle is a complicated process involving the electrical and mechanical phenomena in an apparently independent way (Marmont and Wiersma, '38, Kuffler and Katz, '47, Hoyle and Wiersma, 58b). It is possible that the glutamate induced changes in the myoneural junction are inhibited by nervous activity. The effect of γ-aminobutyrate mentioned above, supports this. However, since the mechanical inhibition acts late in the chain of processes leading to contraction, inhibitability of glutamate induced contractions is to be expected anyway.

Since glutamate seems to cause depolarization of the muscle membrane at the myo-neural junction, it would act like a transmitter substance. Glutamate may not be the natural transmitter, however. The threshold concentration is rather high as compared with that of known transmitter substances. Also there are very large amounts of glutamate present in crustacean muscle (from chromatographic evidence about 1.5 mg/gm), and it seems likely that such large amounts of this amino acid would have other functions than that of a transmitter substance. Furthermore, Hoyle and Wiersma ('58a and b) concluded that transmission at the fast and slow myo-neural junctions is mediated by different compounds. Since glutamate depressed the mechanical and electrical effects of stimulations of both the "fast" and "slow" axon it appears to act on both kinds of myo-neural junctions. It seems possible, however, that the natural transmitter substances are chemically related to glutamate and that the latter imitates their actions at high concentrations.

SUMMARY

The application of glutamate to crustacean muscle caused a contraction accompanied by potential changes. After the contraction which was of short duration the mechanical as well as the electrical effects of stimulation of the "fast" and of the "slow" axons for the adductor muscle of the claw were greatly depressed. Glutamate induced contractions of the abductor muscle could be depressed by stimulation of its inhibitory axon. This compound had no appreciable effect on crustacean nerve. The observations were consistent with the postulate that glutamate acts on the muscle membrane at the myoneural junction, causing first depolarization and then densensitization of the membrane.

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ndom and Oriented Movements of acken Spermatozoids

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n the absence of orienting influences, cken spermatozoids, and many other all organisms, swim randomly in their pending medium in a manner suggest- of Brownian movement. Wilkie ('54) orted that for long time intervals (5 to seconds) the mean square displacement bracken spermatozoids was proportional the time interval, as is the case with wnian movement. Similar results have en reported for protozoa (Przibram, '13, ; Fürth, '20a, b).

The movements of bracken spermato- ds can also be oriented precisely by con- ntration gradients of malic acid salts feffer, 1884) or by voltage gradients rokaw, '57). Under these conditions eir random movements appear to be sup- essed. In this paper the relationship tween oriented and unoriented move- ents of bracken spermatozoids is ex- mined with reference to a new theoretical odel for random movements.

THEORY

We shall consider the following model. small spherical particle is constrained to ve in the x-y plane through a viscous edium. It moves forward with a constant eed, s, large enough that the effect of nslational Brownian movement on its vement is negligible. The direction of vement of the particle varies as its or- ntation is altered by random fluctuations its interaction with the molecules of the scous medium: i.e., by rotational Brown- n movemnt. We are interested in the erage parameters of the paths resulting nen this rotational Brownian movement superimposed on the steady speed, s.

The theory of rotational Brownian move- ent was developed by Einstein in 1906. ø is the angle between the direction of vement of the particle at time t and its direction at $t = 0$, and the changes in ø are random, the probability distribution of ø is given by:

$$P(\phi, t) = 1/2 \left(\frac{\tau}{\pi t}\right)^{1/2} e^{-\phi^2\tau/4t}. \quad (1)$$

τ is the reciprocal of the rotational diffusion coefficient. Einstein used Stokes' law for a sphere rotating at constant velocity to find that:

$$1/\tau = \frac{k\,T}{8\pi\eta\rho^3}, \quad (2)$$

where k = Boltzmann's constant, T = the absolute temperature, η = the viscosity constant for the suspending medium and ρ = the radius of the particle. For any time interval t, the mean-square turn, that is, the expected value of ϕ^2, is:

$$\int_{-\infty}^{+\infty} \phi^2 P(\phi, t)d\phi = 2t/\tau. \quad (3)$$

If the particle is moving in the y direction at $t = 0$, the expected value of its velocity component in the y direction after time t is:

$$s \int_{-\infty}^{+\infty} \cos\phi\, P(\phi, t)d\phi = s\, e^{-t/\tau}, \quad (4)$$

and the particle's expected position component in the y direction is:

$$s \int_{o}^{t} e^{-t/\tau}\, dt = s\tau(1 - e^{-t/\tau}). \quad (5)$$

As the particle exponentially loses its original velocity in the y direction, its movements become random (i.e., less information is available to predict its position), and its expected position after a long time will be a point at a distance $s\tau$ along the y direction from its original position. Its actual position will be distributed randomly about this point. The movement of this model is now formally similar to translational Brownian movement in two dimen-

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sions, and equations for translational Brownian movement (Chandrasekhar, '43) can be applied. The mean square displacement of the particle from its position at t = 0, for any original orientation, is then:

$$\text{Average of } (x^2 + y^2) = 2 s^2 \tau^2 [t/\tau - (1 - e^{-t/\tau})]. \quad (6)$$

For small values of t, this reduces to:

$$x^2 + y^2 = s^2 t^2, \quad (7)$$

and for large values of t, it becomes:

$$\text{Average of } (x^2 + y^2) = 4 D t, \quad (8)$$

where D, the two-dimensional diffusion coefficient for the random movement of this particle, is defined by:

$$D = 1/2\, s^2 \tau. \quad (9)$$

Equations 8 and 9 were checked by measurements of the average value of $(x^2 + y^2)$ on an artificial random track constructed from a table of random normal deviates, using equation 3. Similar equations can be obtained by considering random walk with persistence of direction and extrapolating to zero step length (Fürth, '20a).

In order to introduce a basis for orientation in a gradient, we shall now add to the model the assumption that the particle carries an electric dipole moment, M, parallel to its direction of movement. When an external voltage gradient, grad V, is applied suddenly, the particle will turn against the viscous resistance of the suspending medium, according to the equation:

$$d\theta/dt = -\beta \sin \theta \quad (10)$$

where θ is the angle between the direction of movement of the particle and the direction of the voltage gradient, and

$$\beta = \frac{M \text{ grad } V}{8\pi\eta\rho^3}. \quad (11)$$

Eventually, an equilibrium will be approached where the orienting force is balanced by the tendency for random disorientation. This situation was discussed by Debye ('29) for the case of a polar molecule in a gradient, subject to thermal agitation. The equilibrium is described by Langevin's function:

$$\text{Average of } \cos\theta = \coth z - 1/z, \quad (12)$$

where

$$z = \frac{M \text{ grad } V}{k T}. \quad (13)$$

If T and η are constant, equations 2 and 11 give

$$z = \tau\beta. \quad (14)$$

The particle will move up the gradie with an average velocity of s cos θ.

MATERIALS AND METHODS

The multiflagellate spermatozoids of t bracken fern (*Pteridium aquilinum* (L Kuhn) were obtained as described in previous paper (Brokaw, '58a). For the experiments, the spermatozoids were su pended in a solution containing 0.005 *tris* (hydroxymethyl) aminomethane—h drochloric acid buffer, pH 8.1; 5×10^{-4} calcium chloride and 5×10^{-4} M sodiu L-malate. These experiments were carri out at room temperature, 19 to 20°C.

Photographic records of sperm mov ments were obtained by the "dark-groun track" method (Brokaw, '58b), while t sperm suspension was contained in haemocytometer slide of depth 0.1 m This effectively limits the major portio of sperm movement to a region parall to the film plane and within the depth focus of the optical system. The use a Robot Jr. camera, with a spring motc allowed several exposures to be made rapid succession.

To measure the orientation of sper movement by voltage gradients, sm blocks of agar containing 3 M potassiu chloride were placed on the central raise portion of the haemocytometer slide at t sides of the cover glass over the sper suspension. Silver–silver chloride ele trodes were inserted into the agar block This method of establishing voltage grad ents was substituted for that used in ea lier experiments to ensure that the sper suspension was not contaminated by tox products of the electrode reactions durin the course of an experiment. The condu tivity of the resulting cell was measure when filled with dilute potassium chlorid solutions. The voltage gradient within t cell was about 7% higher than that ca culated by dividing the voltage differenc applied to the electrodes by the width the cover glass; this probably indicate entrance of potassium chloride into t ends of the cell from the agar blocks.

RESULTS

Random movements. The mean-squar displacement of bracken spermatozoid was measured using a technique simil

that used by Wilkie ('54). An eyepiece crometer scale having 1 mm cross-rul-gs was used as a cover glass for the emocytometer slide containing the sperm spension. A timer was started when a ermatozoid swam beneath an intersec-n of the cross-rulings, and the position the spermatozoid relative to this in-section was recorded at a signal from e timer. The time intervals used were nited in length by the width of the emocytometer slide chamber (6 mm). nce this procedure does not give an equal ance of observation to slowly moving ermatozoids, which seldom swim beneath intersection of the cross-rulings, it ould be expected to yield a somewhat gh estimate of the mean diffusion co-icient. The estimate obtained may be ore indicative of the behavior of the ermatozoids under optimum conditions. A total of 413 records, at 7 different ne intervals, is summarized by figure 1. agreement with equation 6, the points e roughly assymptotic to a line which tersects the abscissa on the positive side the origin. According to equations 6 nd 8, this intersection is the point $t = \tau$, nd the assymptote has a sloping of 4 D, ut the results are not sufficiently exten-ve or precise to provide very accurate estimates of τ and D. The curve drawn in figure 1, which was calculated from equation 6 using a value of 1.5×10^5 $\mu^2 \cdot sec^{-1}$ for D and a value of 5 seconds for τ, fits these points satisfactorily. Fürth ('20b) also found that the movements of *Paramecium* agreed with an equation similar to equation 6.

To obtain a more precise estimate of τ, several dark-ground-track photographs of about 4 seconds duration were used to record random movements from 4 samples of sperm suspension. Time marks were inserted into the track records by interrupting the illumination with a rotating vane at intervals of 0.475 seconds. The records were projected onto a screen, and the change in the average direction of sperm movement between each time mark was measured with a protractor. To estimate the swimming speed, s, the distance between time marks was measured on straight sections of tracks.

The results are summarized in table 1, and the distribution of turns is shown in figure 2. The distribution is significantly different from the normal distribution described by equation 1, with $\tau = 3.7$ seconds, mainly because of an excess of large turns. If the 11 turns greater 70° are eliminated, the distribution of the remain-

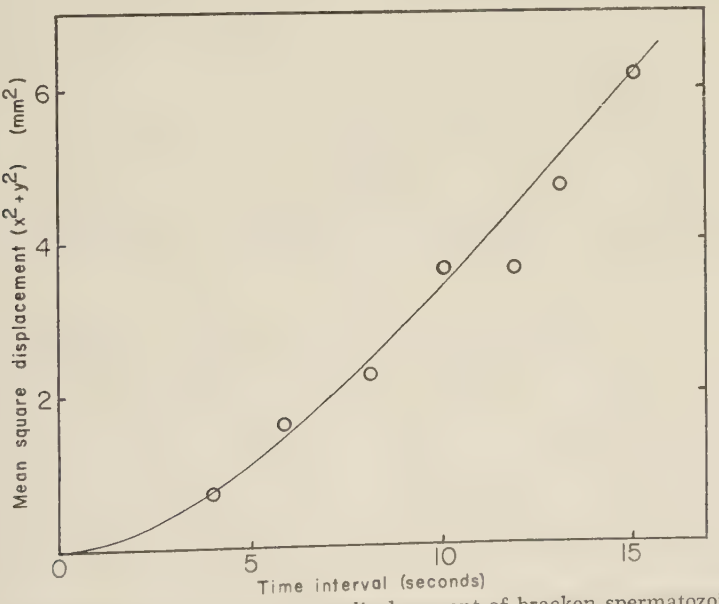

Fig. 1 Measurements of the mean-square-displacement of bracken spermatozoids.

TABLE 1

Measurements of random turns and swimming speed of bracken spermatozoids

Experiment no.	1	2	3	4	Combined results
Number of frames photographed:	4	3	4	3	14
Approximate number of spermatozoids involved:	17	15	8	12	52
Number of measurements of s (on first and last frames):	22	21	12	20	75
Mean value of s ($\mu \cdot sec^{-1}$):	166	205	220	202	196
Total number of random turns measured:	199	95	92	87	473
Root-mean square average turn in 0.475 seconds:	27.4°	30.1°	31.2°	29.1°	29.1°
τ (seconds) calculated from equation 3:	4.2	3.4	3.2	3.7	3.7

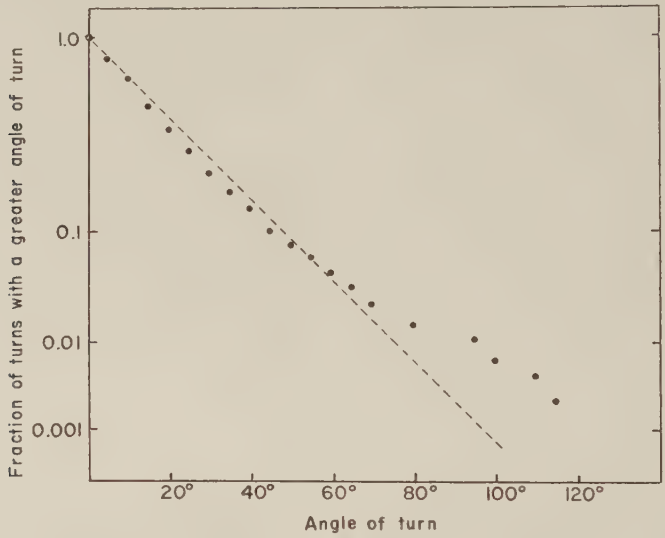

Fig. 2 Distribution of the angles of turn of bracken spermatozoids. Each point indicates the fraction of turns having a greater angle of turn. If the distribution of turns were random in agreement with equation 1, the points should lie on the dashed line, since a probability ordinate scale has been used.

ing 462 turns is more nearly normal, with a root-mean-square average of 25.6°, corresponding to a value of $\tau = 4.8$ seconds. The shape of the distribution of turns may be the result of the distribution of τ in the population of spermatozoids, or it may indicate that equation 1 does not apply strictly to the turns of bracken spermatozoids. This cannot be decided at present, and the value obtained for τ must be accepted as only approximate.

The distribution of measurements of s shown in figure 3. This distribution can b used to obtain an estimate of th mean diffusion coefficient, by averagin $(3.7s^2)/2$, on the assumption that the is no correlation between s and τ. On th basis, $D = 0.8 \times 10^5\ \mu^2 \cdot sec^{-1}$, significant less than the value which agreed with th measurements of the mean-square-di placement.

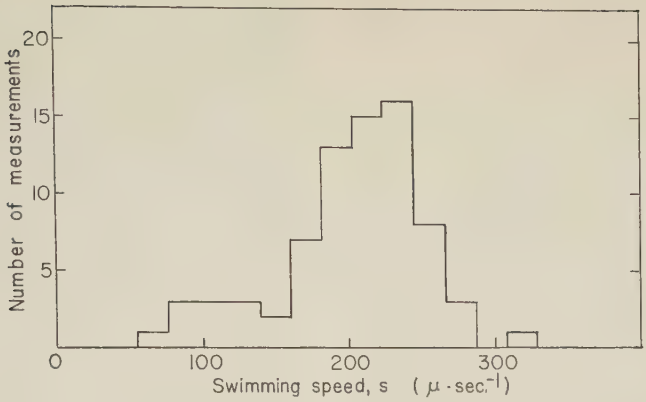

Fig. 3 Distribution of measurements of the swimming speed of bracken spermatozoids. The arithmetic mean speed is 196 $\mu \cdot sec^{-1}$; the root-mean-square speed is 206 $\mu \cdot sec^{-1}$.

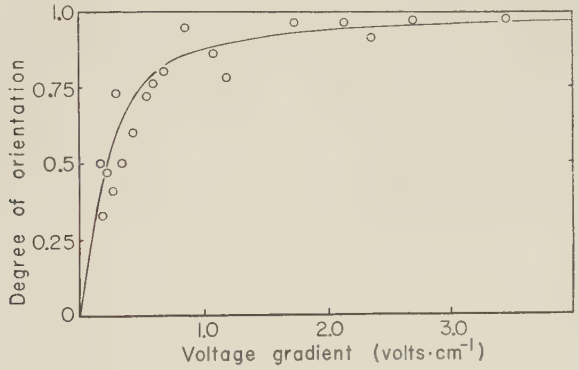

Fig. 4 Measurements of the degree of orientation (average value of cos θ) of bracken spermatozoids in various voltage gradients.

Oriented movements. Voltage gradients were applied to the same 4 samples of sperm suspension used for the above measurements of τ, and, after a few seconds were allowed for equilibration, three ½-second dark-ground exposures were taken in rapid succession. The tracks of a particular spermatozoid were identified on the three successive frames of the resulting records, to determine the direction of movement. The angle θ between the direction of the gradient and the average direction of sperm movement during the ½-second exposure was measured with a protractor. The degree of orientation, i.e., the average value of cos θ, at various voltage gradients, is shown in figure 4. Each point represents the average of about 40 measurements.

In previous work (Brokaw, '58b), the response of bracken spermatozoids to a suddenly applied voltage gradient was found to follow equation 10. For the two spermatozoids studied in that paper, $\beta = 1.5$ grad V rad·sec^{-1}. Further measurements on a larger sample of spermatozoids (Brokaw, '58c) gave a mean value for β of 1.6 grad V rad·sec^{-1}.

This value of β was inserted into equations 12 and 14, and a value for τ was calculated for each of the 19 points in figure 4. These values for τ ranged from 2.4 to 13 seconds, with a mean of 5.1 seconds, in good agreement with the independent estimates of τ obtained from the measurements of random movement. The curve in figure 4 was calculated from equation 12, using $\tau = 5.1$ seconds, showing

that these results agree well with the form of equation 12.

DISCUSSION

The movements of bracken spermatozoids should resemble those of the model presented in the section on theory if they are restricted to two dimensions and if the source of turning is random. The first condition appears to be satisfied in a haemocytometer slide of depth 0.1 mm, even though this is considerably larger than the diameter of a spermatozoid. Collisions with the glass surfaces and projections of inclined helices are only rarely found in the dark-ground-track records of sperm movement. Presumably the glass surfaces affect the hydrodynamic conditions for sperm movement and favor movement in a plane, probably midway between the surfaces.

Agreement of the distribution of turns (fig. 2) with equation 1 would provide the most decisive demonstration of the randomness of turning. The present results are inconclusive because the distribution of the parameter τ in the sperm population has not been studied. Better agreement was obtained when the results were compared with equations 6 and 12.

The disparity between the two estimates of D may, as mentioned, be due to differences in the population sampled. The estimates of τ are in good agreement, in spite of the fact that the measurement of β was performed a year and a half earlier, on spermatozoids grown from spores collected over 300 miles south of the source of later material. On the whole, the movements of bracken spermatozoids are accurately described by a model based on random turns, and orientation to a gradient is merely superimposed on the random movement without intrinsically altering it.

The coiled body of a bracken spermatozoid is approximately the same size as a sphere of radius 5 μ. If the source of random turning were rotational Brownian movement, a value of τ of about 800 seconds would be predicted by equation 2. Since the measured values of τ are very much less, the random turning is clearly not due to rotational Brownian movement of the body. Random turning might result from random variations in the output of the flagella on different sides of th body. As a rough example, if one ha of the body moves more than the oth half, the resulting angle of turn will equ the difference between the linear mov ments of the two halves divided by the c ameter of the body. To give the root-mea square turn measured here, of about 1 radian in a ½-second interval, the va ability in flagellar activity must be gre enough to also cause a 2 to 3% variatic (percentage standard deviation) in th linear distance moved by a spermatozo in ½ second. This is consistent with th records which have been obtained. B cause of the rotation of a spermatozoid a it swims in its helical path, only thos variations which are rapid compared wit the period of one rotation (0.2 to 0.5 se onds) will contribute to the random turn ing. An inspection of records of sperm movement for a correlation between τ an the variance of s might provide a test c this model of the origin of random turns

The two estimates obtained for th diffusion coefficient of bracken spermato zoids, 0.8 and 1.5×10^5 $\mu^2 \cdot sec^{-1}$, are cor siderably larger than the value 0.05 $\times 10^5$ $\mu^2 \cdot sec^{-1}$, reported by Wilkie ('54 In Wilkie's experiments, the sperm sus pension had a depth of about 1 mm, s that the spermatozoids were probably nc constrained to move in two dimension but this difference cannot account fo the difference in results. If the diffusio coefficient were so radically altered b this difference in the depth of the sperm suspension, the sensitivity to a voltag gradient should be similarly altered; thi is not the case. A difference in the op posite direction would be expected if th proximity of the glass surfaces has a del eterious effect on sperm movement; thi was found by Przibram ('18) in his ex periments with *Paramecium*.

Wilkie's treatment of the diffusion o spermatozoids towards an archegonium would not be valid with a diffusion co efficient as large as that measured here since the dimensions of the diffusion field around an archegonium are not large com pared with s_τ.

SUMMARY

1. A theoretical model for the random movements of organisms in two dimen

ons is presented, based on the super-
osition of changes in orientation similar
rotational Brownian movement on a
nstant forward speed. The behavior of
is model in an orienting gradient is
nsidered.

2. The mean-square-displacement, the
ot-mean-square average turn, the swim-
ing speed, and the degree of orientation
a gradient at equilibrium have been
easured for bracken spermatozoids swim-
ing in a thin suspension.

3. The model adequately represents the
ovements of bracken spermatozoids. The
ndom turns cannot result from rotational
rownian movement, but are formally sim-
ar. They are not intrinsically altered in
ie presence of an orienting gradient.

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othschild for suggesting an investigation
f this problem.

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he Mechanism of the Asymmetrical Distribution of ndogenous Lactate about the Isolated oad Bladder[1,2]

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It has previously been observed that the ıctic acid produced anaerobically by iso- ıted frog skin was distributed asymmetri- ally about the skin, i.e., more lactate ac- umulated in the fluid bathing the inside f the skin than in that bathing the outside Leaf and Renshaw, '57). Similarly, under naerobic conditions more lactate accumu- ıted in the medium bathing the serosal urface of the isolated toad bladder than in ıat bathing the mucosal side (Leaf, Page nd Anderson, '59). A high concen- ration of lactate within the mucosal cells f the toad bladder and a mucosal surface f these cells which is relatively less per- ıeable to lactate than is the serosal surface vould explain all the observations regard- ng the asymmetrical distribution of this on.

The purpose of the present study is to how that the concentration of lactate vithin the tissue water was in fact much ıigher than its concentration in the me- lium and to present measurements of the elative permeability of the mucosal and erosal surfaces of the toad bladder to actate. The conclusion will be reached hat a sufficient concentration gradient be- ween membrane and medium and a suf- ıcient difference in permeability of the wo surfaces of the membrane exist to account for the observed asymmetrical dis- ribution.

EXPERIMENTAL PROCEDURE

Lactate was determined in incubating medium and tissue extracts by the method of Barker and Summerson ('41). DL-Lactic-l-C^{14} acid was obtained from Nu-clear-Chicago Corp., Chicago. Measure- ment of C^{14}-labelled lactate in bathing me- dium and tissue extract was accomplished either by plating 500 μl of medium or ex- tract on planchets and counting with a thin window gas flow counter (Automatic Sam- ple Changer Model 750 Baird-Atomic In- struments, Inc., Cambridge, Mass.) or by adding 100 μl volumes of medium directly to vials containing 10 ml of a mixture of toluene plus phosphor (70%) and absolute alcohol (30%) and counting in a liquid scintillation counter (Tri-Carb Liquid Scintillation Spectrometer Packard Instru- ments Co., LaGrange, Illinois). Great care was taken to keep self-absorption constant when using the gas flow counter.

The urinary bladder of the toad, *Bufo marinus*, was used in these experiments. All measurements of the flux of C^{14}-labelled lactate were made under anaerobic conditions obtained by gassing with pure nitro- gen in a chamber similar to that described by Ussing and Zerahn. Membranes were short-circuited and were bathed on both sides by frog Ringer's solutions which were identical except for the tracer amounts of C^{14}-labelled lactate added to one side. The composition of the Ringer's solution was Na, 113.5; K, 1.88; HCO_3^-, 2.38 meq/l and Ca, 0.89 mM. Thirty minutes were al- lowed for equilibration after adding the isotopic lactate before flux measurements were made over two to 4 successive 30-min- ute periods. At the end of the last period the medium was drained off and the tissue rapidly removed from the apparatus, blot- ted on Whatman no. 54 filter paper and

[1] A brief report of this work has appeared (Leaf, '58).

[2] Supported by funds from the John A. Hart- ford Foundation, Inc., the United States Public Health Service (Grant H-2822) and the American Heart Association, Inc.

[3] Investigator, Howard Hughes Medical Insti- tute.

dropped into a tared tissue homogenizer containing 250 μl of 10% trichloracetic acid. After weighing and homogenizing thoroughly, 3.0 ml of Ringer's solution were added, the mixture stirred and then centrifuged. The supernatant fluid was used for tissue lactate analyses and counting. To compare medium and tissue radioactivity, aliquots of medium were diluted with a trichloracetic acid-Ringer's solution to yield the same final concentrations of inorganic ions and trichloracetic acid in medium and tissue dilutions and equal volumes of each were placed on the planchets so that self-absorption would be the same for both. To obtain tissue water content from wet tissue weights a mean figure for tissue dry weight was used, $17.8 \pm 2.4\%$, which had been separately determined in 51 measurements of both the dry and the wet weights of this tissue.

To test the homogeneity of the radioactivity in both medium and tissue, descending chromatography on paper with a solvent system of ether, acetic acid and water in the ratio of 13:3:1 was used (Denison and Phares, '52). Radioactive spots on the paper were localized with a gas flow micromil window strip counter (Actigraph II, Model C 100 A, Nuclear-Chicago Corp., Chicago).

Teorell ('49) has given the quantitative statement for the flux of any species through a membrane in general terms,

$$J = \omega c \frac{d\mu}{dx}$$

in which J is the flux across unit area of membrane per unit time of the substance considered, ω is the mobility of the substance through the membrane, c is its concentration on one side of the membrane and $d\mu/dx$ is the driving force or gradient of chemical potential through the membrane.

$$J = \omega c RT \frac{d \ln a}{dx}$$

As dx, the thickness of the diffusion barrier will not be known in these experiments; it will be incorporated into k,[4]

$$\frac{RT\omega}{dx} = k$$

whose numerator has the dimensions of the usual Fick diffusion coefficient. Hence

$$J = kc \frac{da}{a}$$

but

$$a = fc$$

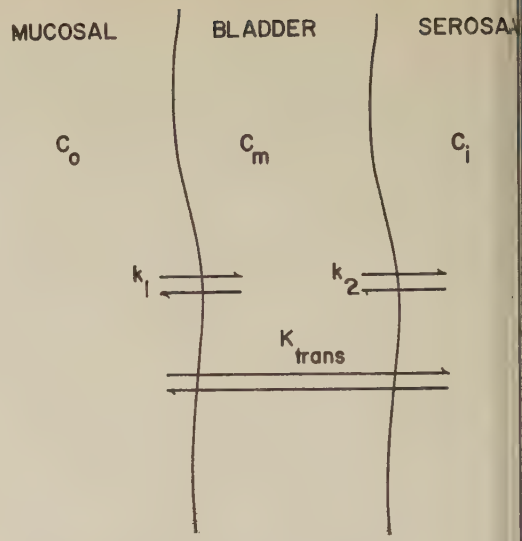

Fig. 1 Schematic representation of a cross section of bladder wall with vertical lines denoting the mucosal and serosal surfaces respectively. C_o, C_m and C_i are the concentrations of any substance considered in the mucosal bathing medium, tissue water and serosal medium, respectively, and k_1, k_2 and K_{trans} are the permeability coefficients through the mucosal surface, serosal surface and across the entire membrane respectively, for this substance.

in which c is concentration and f is the activity coefficient of the diffusing substance. By making the simplifying assumption, which will be considered below, that f is not only the same on the two sides of the membrane but has the same value through the membrane we have

$$J = k\, dc$$

It will be shown later that this approximate solution will suffice for the measurement of the relative permeability of the mucosal and serosal surfaces of the isolated toad bladder.

The rationale of these measurements is perhaps best explained by reference to figure 1. This figure is a schematic represent-

[4] The relationship between k and the usual diffusion and permeability coefficients should be pointed out. To use a diffusion coefficient the distance, dx, must be known and this coefficient has the dimensions of $cm^2\ sec.^{-1}$. The permeability coefficient is used when dx, though not known, may be assumed to be constant and it has the dimensions of $cm\ sec.^{-1}$ (Teorell, '49). k, as used here, has the same dimensions as a permeability coefficient but the assumption that dx is constant is not made. It is this sense that the k values obtained in this study are considered to be relative.

tion of a cross-section of bladder wall with ne two vertical lines denoting the mucosal nd serosal surfaces respectively. k_1 and $_2$ are the respective permeability coefficients of these two surfaces and C_o, C_m, nd C_i are the concentrations in mucosal nedium, membrane water, and serosal nedium respectively of any substance vhose permeation is being studied—in this ase lactate.

When a steady state flux of the substance considered has been set up across he membrane, and if permeation only by imple diffusion is considered

$$C_o - C_i)K_{trans} = (C_o - C_m)k_1 = (C_m - C_i)k_2 \quad (1)$$

Hence

$$C_o - C_m = \frac{(C_o - C_i)K_{trans}}{k_1} \quad (2)$$

nd

$$C_m - C_i = \frac{(C_o - C_i)K_{trans}}{k_2} \quad (3)$$

dding equations 2 and 3 and rearranging, ve obtain,

$$K_{trans} = \frac{k_1 k_2}{k_1 + k_2} \quad (4)$$

By adding a tracer amount of radioactive C^{14}-labelled lactate to the medium bathing ne surface, say the mucosal side, and neasuring its rate of appearance on the pposite side, K_{trans} may be directly evaluated over successive periods. After several periods during which the constancy of the flux rate indicated that a steady state had been approximated, the experiment was terminated, and the membrane was rapidly weighed and homogenized in 10% trichloracetic acid. The concentration of C^{14}-labelled lactate in tissue water was obtained by measuring the radioactivity of the supernatant tissue extract (see below). From the concentration of C^{14}-labelled lactate within the membrane water and its rate of appearance in the serosal bathing medium, k_2 could likewise be evaluated. By substituting values for K_{trans} and k_2 into equation 4, k_1 may then be calculated. Because of the relatively low permeability of the membrane to lactate, C_o remained so much larger than C_i during an experiment that back diffusion could be ignored. k_1 and k_2 may, of course, be evaluated by the same procedure as readily in the opposite direction.

RESULTS

Table 1 compares the concentration of lactate ion in tissue water and in the medium bathing the mucosal and serosal surfaces. The values were obtained at the end of 60 to 90-minute periods of incubation anaerobically. It is evident that the concentration in tissue water considerably exceeds that in the medium. The higher concentration of lactate in the serosal than in the mucosal bathing medium previously noted is again evident. A comparison of the mean concentrations indicates that the tissue water concentration was some 12-fold greater than the concentration in the serosal medium. Thus a considerable concentration gradient exists for lactate between tissue and medium.

Table 2 shows the mean values obtained for k_1 and k_2 when measurements were made from mucosal to serosal sides in 27

TABLE 1

A comparison of the concentration of lactate in tissue water and in mucosal and serosal bathing media following 60 to 90 minutes of anaerobic incubation

Exp.	Lactate concentration[1]		
	Mucosal	Serosal	Tissue water
1	0.03	0.11	1.99
2	0.06	0.12	3.16
3	0.01	0.10	0.43
4	0.00	0.09	1.00
5	0.01	0.10	1.29
6	0.00	0.05	1.94
7	0.07	0.05	1.53
8	0.03	0.08	2.05

[1] In μmole per milliliter.

TABLE 2

Mean values for permeability coefficients for C^{14}-labelled lactate through the isolated toad bladder[1]

k_1	k_2	K_{trans}
A. C^{14}-labelled lactate placed on mucosal side		
5.72 ± 0.62 (27)[2]	66.3 ± 9.1 (27)	5.08 ± 0.36 (27)
B. C^{14}-labelled lactate placed on serosal side		
5.11 ± 0.59 (22)	82.6 ± 12.8 (20)	4.62 ± 0.47 (22)
Difference of means, A—B		
0.61 ± 0.59	-16.3 ± 15.8	0.46 ± 0.59

[1] All values are means plus or minus the standard error of the mean and are expressed as cm $sec.^{-1} \times 10^{-7}$.

[2] Figures in parentheses give the number of 30-minute periods upon which each mean value is based.

periods of 30 minutes each in 7 separate experiments and from serosal to mucosal side in 22 periods of 30 minutes each in 8 other experiments. In every period k_2 was found to be significantly greater than k_1. Note that any C^{14}-labelled lactate from the medium adherent to the mucosal surface of the bladder will be measured as tissue lactate and tend to make k_2 falsely too low while C^{14}-labelled lactate from the medium adherent to the serosal surface will result in underestimation of k_1. Furthermore, because of the variability of these measurements from one bladder to the next and the inability with a single isotopically labelled molecule to make the measurements simultaneously in both directions across the same membrane there was considerable scatter in the values for individual experiments as indicated by the standard errors of the means. In spite of these expected limitations in the experimental procedure, however, satisfactory agreement was found for the values of k_1 and k_2 respectively when measured in the two directions across the membrane.

In order to determine whether the measurements of the concentration of C^{14}-labelled lactate in the tissue water were valid it was necessary to determine whether the radioactivity in the tissue was still associated with lactate. This was done by chromatographic separation on paper of the tissue extract and of the medium at the termination of an experiment. For both medium and tissue extract a single peak of radioactivity was found with the same R_f as that of the lactate. This supports the assumption that all the tissue and medium radioactivity was in fact lactate.

Lactate produced endogenously is presumed to be formed within the cells of the membrane, largely the layer of mucosal epithelial cells. Measurements were made of the distribution in the membrane of the exogenously supplied C^{14}-labelled lactate in order to determine whether it had access to the same zones within the tissue as did the endogenously formed lactate. In 6 experiments bladder tissue was incubated anaerobically in Ringer's solution to which C^{14}-lactate had been added. After 60 minutes of incubation tissue was removed and the C^{14}-labelled lactate concentration in tissue water and medium determined. The results showed that the concentration of radioactively tagged lactate in the tissue water was 93, 114, 106, 104, 119, and 99% respectively of its concentration in the medium. Hence diffusion equilibrium had been approximated between tissue water and medium. These results are compatible with the view that exogenous lactate has access to all portions of the cell and that the higher concentrations of endogenous lactate in the tissue than in the medium are not the result of any process by which lactate is actively accumulated by the cells.

DISCUSSION

The much higher concentration of lactate in tissue water than in the bathing medium and the difference in permeability between the two surfaces of the membrane probably provides an adequate explanation for the asymmetrical distribution of lactate observed about the isolated toad bladder. Because k_1 and k_2 represent merely relative permeability coefficients for the diffusion barriers presented to lactate at the two opposite surfaces of the membrane certain assumptions have been permitted in their evaluation which would not be justifiable if absolute values for these coefficients were sought. In order that the limited significance of the relative permeability coefficients be understood these assumptions should be scrutinized.

Concentrations of lactate in the medium expressed as counts per unit volume were used instead of activities for simplicity. As the concentration of lactate was 10^{-3} molar at its highest, replacing activity by concentration probably introduces negligible error. The assumption that concentration may replace activity within the tissue water is less justifiable. The evidence cited from chromatography of the tissue extract indicates that the radioactivity within the membrane remained in the lactate but tells nothing about the chemical activity of such lactate. However the finding that the concentration of exogenous lactate in tissue water at diffusion equilibrium approximates that in the medium suggests that the activity coefficient of the lactate in the tissue is not significantly different from that in the medium. If the activity coefficients for lactate in the diffusion barriers were different from its

lue elsewhere in the membrane or me-um, the effect of this would be incor-rated into the relative values for k_1 and .

It has been assumed that the flux meas-rements were made under steady state nditions in order to justify the use of quations 1 through 4 for calculating k_1 nd k_2. Actually the experimental con-itions only approximate steady state con-itions as the diffusion of labelled lactate cross the membrane must result in a radual fall in its concentration on the igh side" and rise in its concentration in he "low side." This in turn would result n a continuously changing concentration f labelled lactate in the tissue water. How-ver, during the time of an experiment the oncentration of C^{14}-lactate in the medium n the opposite sides of the membrane naintained a difference of at least 1000-old and as the decline in its concentration n the "high side" was much less than % during an experiment, the slight devia-ion from true steady state conditions will ot significantly affect the present results.

It has been assumed for the purpose of his treatment that the membrane is ho-nogeneous in so far as the two diffusion arriers were depicted in Figure 1 as uni-orm over the two surfaces of the mem-rane. It seems possible that the mucosal urface of the epithelial cells is entirely mpermeable to lactate while the serosal urface of these cells is permeable to lac-ate. In such a situation lactate could pene-rate the mucosal barrier only between ad-acent cells while it could enter or leave he cells only along their serosal surface. Such a picture of the membrane would also e compatible with the finding that at equilibrium the concentrations of exogen-ous lactate in medium and tissue water were identical. The present results would e consistent with either view and the elative values of k_1 and k_2 cannot tell us whether the permeability of the two sur-aces is a single-valued function, a variable unction or a discontinuous function with espect to position on the surface.

The permeability coefficients are ex-pressed in centimeters per second instead of square centimeters per second as is cus-omary for diffusion coefficients. This is ecessary because the thickness of the dif-fusion barrier is as yet unknown. The dif-ference between k_1 and k_2 could be attrib-uted to a difference in structure of the opposite surfaces of the membrane, to dif-ferences in thickness of diffusion barriers of similar structure, or even to a difference in the effective surface area of the two faces of the bladder. This limitation does not affect the relative functional magni-tude of k_1 and k_2 which is what has been measured simultaneously in the same membrane. The structure of this mem-brane as viewed by electron microscopy currently being investigated by Dr. Alan Cohen indicates that differences in thick-ness or in total surface area of the oppo-site surfaces of the cell membrane probably cannot account for the present findings.

The justification for assuming the exist-ence of only two diffusion barriers in the membrane to lactate is that this constitutes the minimum requirement. Lactate formed within the mucosal cells must pass through at least the two opposite surfaces of the cells to reach the bathing medium on each side. As the mucosal border of the cell con-stitutes the mucosal surface of the mem-brane and as the supporting connective tissue and serosa in apposition to the serosal border of the mucosal layer of cells appears of such loose structure, k_1 and k_2 may tentatively be identified anatomically with the two opposite faces of these mu-cosal cells.

The generalized relationship of the trans-membrane permeability coefficient, K_{tran}, across a membrane with n separate dif-fusion barrier with permeability coeffi-cients k_1, k_2, k_3 . . . k_n is[5]

$$K_{trans} = \left(\frac{1}{k_1} + \frac{1}{k_2} + \frac{1}{k_3} + \ldots + \frac{1}{k_n} \right)^{-1} \quad (5)$$

The requirement for passive diffusion across any barrier is that the respective value of k be the same in both directions. If any process in the membrane facilitates the movement of the test substance in any manner in one direction across the diffu-sion barrier, this distinction must be recog-nized, and the resulting different values of

[5] In the preliminary report (Leaf, '58) this equation was given incorrectly. For a simple sys-tem with only two diffusion barriers, however, both equations simplify to the form, $K_{trans} = k_1k_2/(k_1 + k_2)$.

k in the two directions must be used in equations 4 or 5.

In the present study all measurements were made across the short-circuited bladder so that no electrical potential existed across the membrane and the expectation for an ion which moves passively through the membrane is that its permeability coefficient will be equal if measured in either direction across the membrane. The equal values for K_{trans} shown in table 2 for the two directions across the membrane indicate that lactate fulfills this requirement for passive diffusion. The additional finding that k_1 and k_2 are also equal in the two directions is not a necessary consequence of the condition imposed on the membrane that no electrical potential exists across it. Any residual electrical potential difference remaining in the short-circuited membrane between the medium and the inside of the mucosal cells might influence the permeability in the two directions across the diffusion barriers for any charged particle. k_1 and k_2 could differ considerably in the two directions across the membrane depending only on the residual potential difference and the charge on the diffusing particle, although K_{trans} in the two directions would be equal. That k_1 and k_2 are found not to differ significantly in the two directions suggests that: (1) no residual potential gradients exist between medium and inside of cell in the short-circuited membrane, (2) lactate passes through the diffusion barriers largely as the undissociated lactic acid molecule which would be unaffected by a potential gradient, or that (3) the mucosal surfaces of the cells are in fact impermeable to lactate which only crosses this barrier through channels between the cells so that any potential gradient across the mucosal surface of the cells would not affect the movement of lactate through the membrane. Until measurements of potentials across the mucosal cell surfaces have been determined and more information is available regarding the pathways of passive diffusion through the membrane no choice can be made betwee these possibilities.

SUMMARY

An explanation has been sought for th observation that lactate accumulates asyn metrically about the isolated toad bladd anaerobically; more accumulates in th medium bathing the serosal than in th bathing the mucosal surface. A muc higher concentration of lactate has bee demonstrated in the tissue water than i the medium. Using C^{14}-labelled lactat measurements of the permeability of th two surfaces of the membrane have bee made. These two surfaces are tentativel identified with the opposite faces of th single layer of mucosal cells comprisin this membrane. The permeability to la tate of the serosal surface was found o the average to be some 14-fold greater tha the permeability of the mucosal surfac The observed concentration gradient be tween tissue water and medium and th difference in permeability of the two su faces of the membrane are considered t account for the asymmetrical distributio of lactate that has been noted. The equa ity of the permeability coeffcients as meas ured in the two directions across the mem brane demonstrates the passive nature o the movement of lactate through this mem brane.

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Basic Rhythmicity in the Mitotic Rate of Urodele pidermis and the Difference in Mitotic Rate etween Larvae Reared in the Laboratory nd in a Pond

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Periodic rhythms have been described a number of physiological processes both plants and animals. The phenomon of periodicity in the rate of cell divin was first reported by Fortuyn-van yden ('17, '26) who established definite ythms in mitotic activity in a number of tissues. Similar results have been reted by Ortiz-Picón ('34), Carleton ('34), oper and Franklin ('40) and Bullough 8) on mouse epidermis; by Blumenfeld 9), Babick ('51) and Halberg et al. 4) on rat epidermis; by Cooper and hiff ('38), Broders and Dublin ('39) in man infant prepuce and by Scheving d Gatz ('55) in adult human epidermis. amphibian tissues, Möllerberg ('48) reted this same cyclic phenomenon in the idermis, and Meyer ('54) in the cornea frog larvae.

Salamander epidermis has been used exsively as a convenient "test tissue" in any studies of the mitotic process. These udies include the effects of underlying afts, starvation and feeding, etc. on totic stimulation or inhibition (Overton, , '57). Numerous investigations have en made of the mitotic phenomena asciated with epidermal wound healing d regeneration (Lash, '55; Weber, '57). vestigations of this type have dealt with itotic indices, but usually with little or consideration of a rhythmic or cyclic anifestation of the normal mitotic proc-s. For instance, DiBerardino ('55), reting on mitotic frequency in the epirmis of normal and regenerating tail s of frog larvae, makes no mention of e time of day when tissues were obned for the study.

Evidence is presented in this report that a basic rhythm or cycle does exist in the normal mitotic rate in salamander epidermis over a 24-hour period and that this basic rhythm is maintained under different ecological conditions.

MATERIALS AND METHODS

Two groups of *Amblystoma punctatum* larvae from different environments were used in this study: (1) "Laboratory" animals which were collected in the egg stage and reared in the laboratory until the chronological age of 6 weeks after hatching and (2) "Pond" animals which were collected as larvae from the same pond as were the eggs, and at approximately the same stage of development as the laboratory animals when used in this study. Groups of 20 animals from each environment were sacrificed every two hours over a 24-hour period and fixed in Bouin's. A strip of epidermis, excised from the dorsal lateral body wall of each animal, was prepared histologically as a whole mount and stained with iron hematoxylin. In each specimen, a minimum of 5000 cells was counted and the number of mitotic figures recorded. The mitotic index was calculated as the number of mitotic figures per 1000 cells. The data for each group were statistically analyzed to determine whether the recorded peaks of mitotic activity were significantly different from the lowest periods of activity.

OBSERVATIONS AND DISCUSSION

As seen in figure 1, laboratory animals manifest three peaks of epidermal mitotic

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activity during the 24 hour period. A mean mitotic index of 0.85 occurs between 12:30 and 2:30 P.M., followed by two peaks of smaller magnitude at 8:30 P.M. and 2:30 A.M. with means of 0.45 and 0.50 respe tively. Three peaks of mitotic acitivity al occur in the epidermis of the pond an mals: one at 2:30 P.M. with a mean of 9.

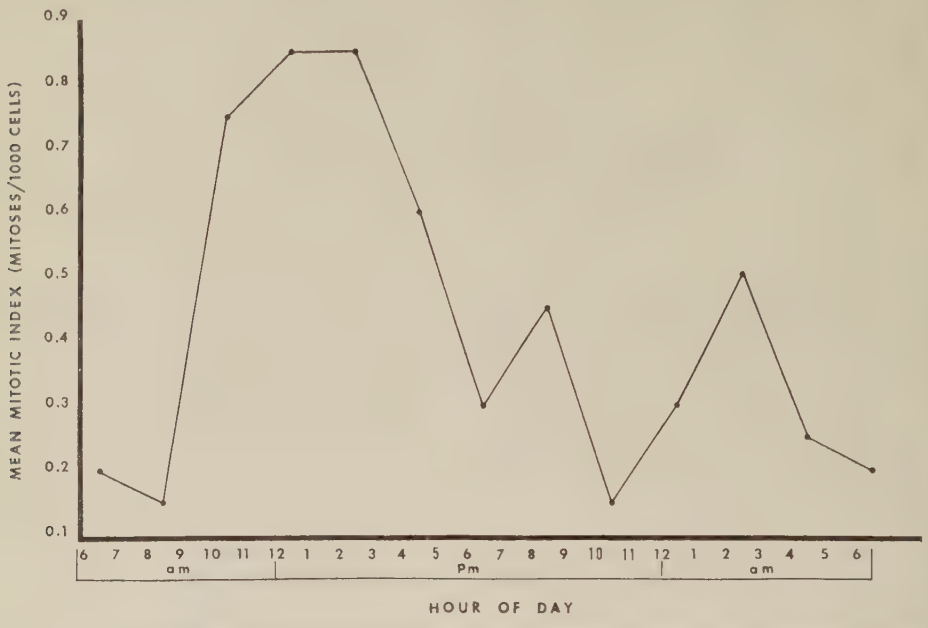

Fig. 1 Mean indices, at two-hour intervals over a 24-hour period, for epidermal mitotic rate in laboratory animals.

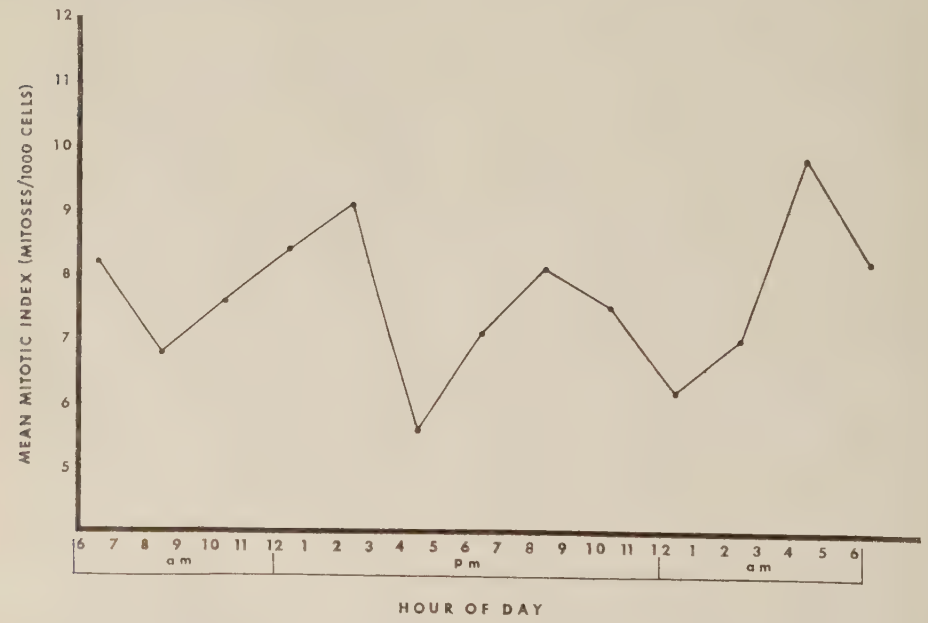

Fig. 2 Mean indices, at two-hour intervals over a 24-hour period for epidermal mitotic rate in pond animals.

other at 8:30 P.M. with a mean of 8.1; d a third at 4:30 A.M. with a mean of 3 (fig. 2). Upon comparing the data for e two groups, the one significant feature ted is the difference in the magnitude the mitotic activity. The graphs of the itotic indices of the two groups, plotted the same scale (fig. 3), point up the ct that overall mitotic activity in the nd group is approximately 20 times as eat as that in the epidermis of the lab- atory group.

It is evident from our observations (figs. 2, 3) that mitotic activity in the epi- rmis of each group is characteristically clic. In both groups the three peaks of idermal mitotic activity occur at approxi- ately the same time and at 8-hour inter- ls.

When the mean mitotic indices of the ree maximum periods of the laboratory animals are compared with those of the three lowest periods for this group, there is a statistically significant difference ($p = 0.001$). A significant difference of the same degree is not obtained when the same analysis is applied to the data for the pond group. Because of the similarity in the form and phase relationships of the curves for the two groups, we believe that the mitotic activity in the epidermis of each group is rhythmic and that similar or identical factors operate at corresponding intervals of time.

The individual variation in the pond animals was greater than that of the laboratory animals, and consequently higher standard deviations were obtained. This wide variation in the pond group is probably due to the adaptation of the organism to the more variable factors in the natural environment and thus illustrates the modi-

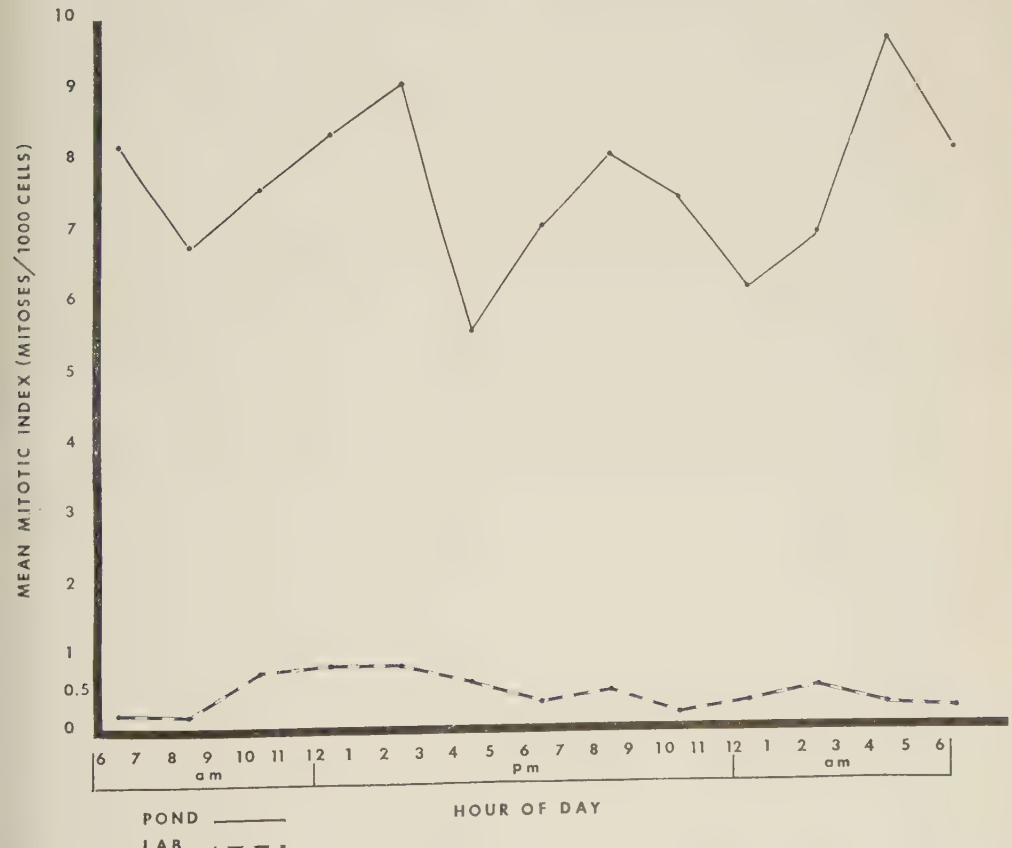

Fig. 3 Comparison between epidermal mitotic indices of laboratory and pond animals plotted on the same scale.

fiability of the mitotic rate, with retention, however, of the basic rhythm.

The only other report that we could find in the literature on rhythmic mitotic activity in amphibian epidermis was that of Möllerberg ('48). He reports peak mitotic activity in the epidermis of *Rana temporaria* to occur between the hours of 12:30 noon and 3:00 P.M., a time when great activity is present in both groups of animals used in our investigation. Although not emphasized in his report, Möllerberg's graphs show two minor peaks at approximately 8:00 P.M. and 2:00 A.M., which correspond in time to our second and third peaks (figs. 1, 2). Möllerberg's investigation is based on 24 larvae, one for each hour of the day, and the conclusion is made that greater mitotic activity occurs during the daytime and the least mitotic activity occurs at night. Our results with the laboratory group confirm his observations. However, the reverse is true in the pond group, as overall activity is slightly higher at night.

Meyer ('54), also using *Rana temporaria* larvae, established a definite rhythm in the mitotic rate of corneal epithelium. His results, as Möllerberg's, are based on few larvae, only one for every two hours over a three-day period. The results of Möllerberg on epidermis differ from those of Meyer on cornea however, in that the reported peaks of activity in the two tissues differ. Whereas Möllerberg found the peak of epidermal mitotic activity during the day, Meyer reports the peak of corneal mitotic activity during the night.

Our observations and results present two questions or problems in need of explanation: (1) What is the cause of the endogenous rhythm? and (2) What are the causes or factors giving rise to the difference in magnitude between the mean epidermal mitotic activity of the two groups?

No satisfactory answer or explanation as to the cause or mechanisms involved in the rhythmic pattern of mitotic activity can be obtained from our observations. We are compelled to agree with Brown ('58) who states, "Much has been learned as to the properties, including modifiability of endogenous rhythmicity. The fundamental problem, however, that of the timing mechanism of rhythmic periods, has largely eluded any eminently reasonable hypothesis in terms of cell physiology or biochemistry."

In attempting to answer the second question, a number of factors can be considered which might influence the magnitude of mitotic activity and thus cause the difference manifested between the two groups. At this time they can only be speculative although systematic investigation of each factor is necessary and is being planned. The difference in physical activity of the animals in the two groups may play a role in creating a difference in mitotic rates. The natural environment of the pond animals may force greater physical activity under harsher physical conditions than the restricted "glass container" environment of the laboratory animals. Perhaps these pond life conditions result in increased epidermal "wear and tear," with greater cell death and consequently the evocation of a greater rate of cell replacement (mitosis) through the initiation of the regenerative processes. Diet may play an important role as laboratory animals were fed solely on enchytraeid worms, a diet which may be deficient when compared to the varied diet of worms, insect larvae, small crustaceans, etc. of the pond animals. The filtered fresh water of the laboratory environment undoubtedly differed in chemical constituents, salt concentration and accompanying osmotic relationships from the semi-stagnant water of the pond environment which contained growing plant and animal forms, more nitrogenous wastes decaying vegetation and animal bodies, and which received drainage from the surrounding soil. These differences in the water could thus affect various aspects of cell metabolism including cell division.

The rate of growth of the two groups of animals may be another factor to be considered. Measurement of body length of the animals in the two groups revealed a mean difference between them of only 3.4 mm, with the pond animals averaging 19.0 mm and laboratory animals averaging 15.6 mm. However, there was considerable overlap in size between animals of the two groups, but no overlap in the ranges of mitotic indices. Gross examination of morphological characteristics, such as de-

elopment of the tail fin, limbs, and gen-ral appearance indicated that the animals ere approximately at the same stage of evelopment. This does not preclude the ossibility, however, that the rate of growth f the pond animals was greater than that f the laboratory animals, and that there-ore some of the difference in mitotic rate etween the two groups may be attributed o the difference in their respective growth ates.

It is doubtful that any one of the factors nentioned thus far, operating alone would necessitate a daily requirement of 20 times s much mitotic activity. However, one noticeable morphological difference was he greater length and development of the ond animals' gills over those of the lab-ratory animals. Several reports relate gill levelopment to oxygen tension of the me-lium. Balák ('07a, '07b), Drastich ('25), nd Bond ('57) report that larvae exposed o a medium with low concentrations of oxygen develop larger gills than larvae naintained in media of high oxygen con-entrations. Medawar ('47) and Bullough nd Johnson ('51) studying *in vitro* growth f rabbit and mouse epidermis respectively, oncluded that the rate of mitotic activity aries directly with the oxygen tension of he medium over a fairly wide range.

The recorded differential in the overall nean mitotic rates, coupled with the ob-served variation in gill development be-ween our laboratory and pond groups, trongly suggests the possibility that a dif-erence in the oxygen tension of the water nvironments may be an important factor n bringing about the difference in magni-ude of mitotic activity between the ani-mals of the two groups.

Bond ('57) presented evidence that mitotic activity of the gill epidermis of the salamander larvae is enhanced by low oxy-gen, although she did not find that an environment rich in oxygen necessarily re-duced mitotic actiivty. She suggests that this maintenance of mitotic activity might be due to a necrotic effect of high oxygen concentration with the consequent initia-tion of the regenerative processes. Bond demonstrated no relationship between the rate of mitosis in the tail fin epidermis and oxygen concentration of the medium, but this particular phase of the investigation, we believe, needs further analysis. Con-templated experiments, in which oxygen tension of the medium will be altered in the laboratory, may help to show the re-lationship of the oxygen tension of the water to mitotic activity of the epidermis and other tissues.

Although the basic questions as to the cause of rhythmicity in the mitotic activity of salamander epidermis and the factors operating to cause a considerable differ-ential in the magnitude of mitotic activity between pond and laboratory remain un-answered and in need of further investiga-tion, the results of this investigation do demonstrate the existence of a basic perio-dicity or rhythm in the mitotic processes of salamander epidermis. This periodic na-ture of the mitotic rate may be of some import in investigations involving mitotic indices or mitogenic factors.

For example, during regeneration of epi-dermis (or other tissues), where regenera-tive processes are interpreted or explained in terms of division rates, is the periodicity of the mitotic process maintained and the increase in mitotic rate manifested during regeneration merely superimposed on it, or is the cyclic nature of the mitotic process lost? Furthermore, the source of animals for experimental work involving mitotic in-dices should be considered. How do ex-perimental results based on animals with an altered mitotic rate because of being reared in unnatural laboratory conditions compare with results obtained on animals which are from the natural environment and which may manifest a different, per-haps more normal mitotic rate?

SUMMARY

1. The rate of mitotic activity in the epi-dermis of *Amblystoma punctatum* larvae is characteristically cyclic for both pond and laboratory animals. Three peaks of mitotic activity occur, between 12:30 noon and 2:30 P.M., at 8:30 P.M. and between 2:30 and 4:30 A.M.

2. The magnitude of the rate of mitotic activity during one 24-hour period is 20 times greater in animals obtained from the natural environment (pond) than in animals reared in the laboratory.

3. Differences in the physical activity, diet, chemical constituents and oxygen

tension of the water medium, and morphological stage of development and rate of growth between the two groups of animals are suggested as possible factors, in need of further investigation, which may be responsible for the difference in magnitude of the mitotic rate between the two groups.

4. Because of obvious differences in the development of the gills of the two groups, the possibility of differences in environmental oxygen tension as a factor influencing the magnitude of mitotic activity is emphasized.

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The Effect of Temperature on the Non-luminescent Oxidation of Cypridina Luciferin[1]

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The blue luminescence of the ostracod crustacean, *Cypridina hilgendorfii*, is produced externally in the sea water by the ejection of an enzyme, luciferase, and its substrate, luciferin. These, in the presence of dissolved oxygen, give rise to the luminescent reaction. (Harvey, '17).

Although the enzyme is relatively stable, the luciferin is very susceptible to oxidation, especially at elevated temperatures (Anderson, '36), but no detailed study of the effect of temperature on the non-luminescent oxidation of luciferin has yet been made. Although the luminescent reaction itself requires oxygen, the luciferin can evidently be oxidized whether or not the enzyme, luciferase, be present. The two reactions are probably not the same, however.

Chase and Lorenz ('45) undertook to study the effect of temperature on this non-luminescent oxidation of luciferin in the presence of the luciferase-catalyzed luminescent reaction. They did this by measuring the luminescent reaction at 5 temperatures, from 10° to 35°C, analyzing the resulting data in terms of an equation for two concurrent first order reactions. One was assumed to represent the enzyme-catalyzed luminescent oxidation of luciferin and the other its non-luminescent oxidation. The equation had the following form,

$$x = \frac{k_1 a}{k_1 + k_2}\left(1 - e^{-(k_1 + k_2)t}\right),$$

where a represented the concentration of luciferin initially present, always the same in each experiment, and x the concentration of the end product of the luminescent reaction, proportional to the total amount of light produced. The rate constant for the luminescent reaction was represented by k_1 and that for the non-luminescent oxidation of the luciferin by k_2, while t was the time, in minutes, from the start of the reaction. It was assumed that the sole effects of temperature were upon the rates of the non-luminescent oxidation of luciferin and upon its luminescent oxidation catalyzed by the luciferase.

The above equation fitted the experimental data remarkably well, as is shown in figure 1, reproduced from the paper of Chase and Lorenz. It was, consequently, possible to calculate values for the rate constants of the luminescent reaction and of the non-luminescent oxidation of luciferin for the various temperatures studied. Figure 2 shows the resulting calculated values of k_1 and k_2 plotted against temperature. It is evident that the temperature optimum for luciferase activity, as obtained in this way, is in the neighborhood of 23°C, unusually low for an enzyme.

Later Chase ('48), studied this same luminescent reaction at different pH's and it became apparent that the non-luminescent oxidation of *Cypridina* luciferin—while a rapid process—was not as rapid as had earlier been supposed, at least in the physiological pH range. Johnson, Eyring and Polissar ('54, pp. 153–159) also noticed the discrepancy and called attention to it, suggesting an alternative mechanism for the effect of temperature.

It became evident that a study of the effect of temperature on the isolated, non-luminescent oxidation of *Cypridina* luciferin was necessary, and it is the purpose of this paper to describe such experiments and their results.

MATERIALS AND METHODS

Under certain conditions the total light obtainable from the reaction of *Cypridina*

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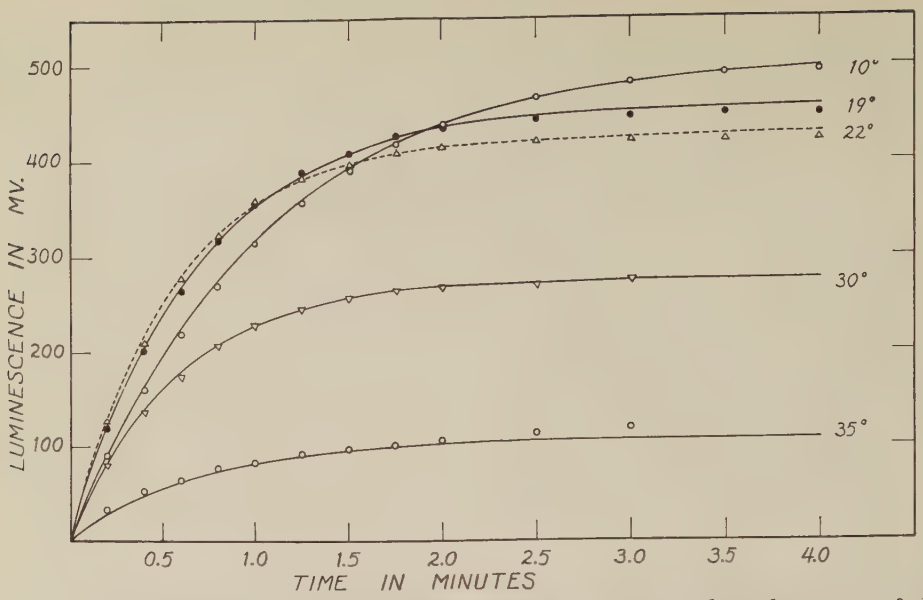

Fig 1 These curves, from the paper by Chase and Lorenz ('45) show the course of the luminescent reaction when constant amounts of luciferin and luciferase react, in pH 6.8 phosphate buffer, at the temperatures indicated. Both the form of the curve and the total light emitted vary with temperature.

luciferin and luciferase is proportional to the concentration of luciferin. It should, therefore, be possible to determine the amount of luciferin remaining in a solution during exposure to various temperatures by removing a sample of the solution at various times, adding luciferase under standard conditions, and measuring the total amount of light produced.

The total light measurements were made by a method based in principle on that described by Anderson ('33), where the luminescent reaction takes place in front of a photoelectric cell. The total output of the photocell accumulates in a condenser whose charge is measured periodically by means of a potentiometer, using a Lindemann electrometer as a null-point indicator. When charge (proportional to emitted light) is plotted against time, the *height* of the resulting curve represents the *amount* of light emitted and the *slope* of the curve is proportional to the *intensity*, of no importance in the present case.

Actually, the apparatus used in the present experiments involved a recording system so that the curves representing the increase in amount of light with time were drawn automatically on paper. The essential components of this apparatus were an R.C.A. 935 photoelectric cell, a highly insulated condenser, a Model 210 Keithley electrometer, and an Esterline-Angus recording meter of 1 milliampere sensitivity.

The luciferase was prepared by the method of McElroy and Chase ('51), Fraction II of their purification procedure being used.

Because it seemed likely that the rate of non-luminescent oxidation of luciferin might be affected by traces of impurities present, two totally different luciferin preparations were employed. One was a methanol extract. The dried organisms were ground and the resulting powder extracted in a Sohxlet apparatus during 24 hours with two charges of benzene, which removed much of the lipid present. After freeing the powder of the residual benzene, it was extracted with absolute methanol for 24-hours in an atmosphere of pure hydrogen. The solid material was then removed by rapid centrifugation and the clear supernatant stored under hydrogen.

The other luciferin solution was prepared by the method of Tsuji ('55), which results in a considerably greater degree

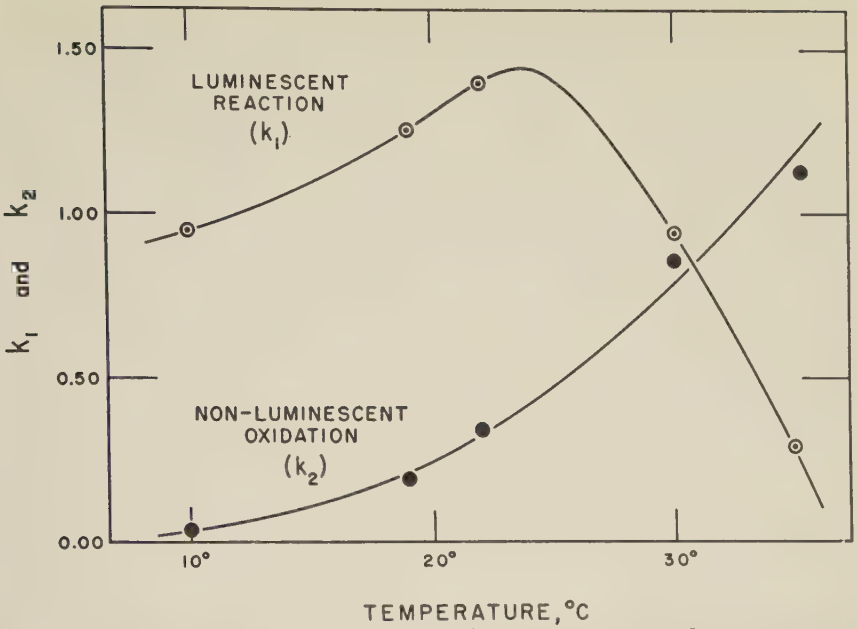

Fig. 2 Effect of temperature on the relative first order reaction velocity constants of the luminescent reaction and the non-luminescent oxidation of luciferin, as calculated from the equation used by Chase and Lorenz ('45).

f purity. The final material is in n-butanol, stored under hydrogen.

The rate of loss of active[2] luciferin was tudied at the following temperatures; 6°, 31°, 36°, 40° and 46°C. Temperature constancy was maintained with a large vater bath, thermostatically controlled. The reaction mixtures containing the luciferin were immersed in the bath in corked lasks.

The procedure for a typical experiment vas as follows. A 100 ml volume of 0.2 M phosphate buffer of pH 6.8, consisting of 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, and 0.01 M NaCl, was placed in a flask in the water bath and allowed to come to the bath emperature. A predetermined amount of ctive luciferin in either methanol or utanol was then added and the solution horoughly stirred up. Two minutes after dding the luciferin a 5-ml sample of the olution was withdrawn and put into the eaction vessel of the light-measuring apparatus. Luciferase in 10 ml of an identical phosphate buffer at room temperature was immediately added. The concentration of the luciferase was such that the otal light, representing the active luciferin, would be emitted in about 10 seconds.

Assays of active luciferin remaining in the reaction mixture in the water bath were made as frequently as was necessary, and were continued until the graphic record of the Esterline-Angus milliameter indicated at least 80% of the reaction over.

The rate constant for the oxidation of the luciferin under these conditions was determined on the assumption that it was a first order reaction, which indeed turned out to be the case. The Briggsian logarithm of the total light emitted for each sample was plotted against the time elapsed since the luciferin had been added to the buffer solution in the water bath. A reasonably good straight line could be drawn through the points and its slope represented the

[2] The term, "active," is used for convenience to designate that form of luciferin which gives bright luminescence in the presence of a high concentration of luciferase although luciferin does not, of course, possess activity in the ordinary sense. Reversibly oxidized luciferin (Anderson, '36) can apparently give a dim luminescence in the presence of luciferase under certain conditions, but this is not characteristic of luciferin in its reduced, or "active," condition.

first order reaction rate constant for the oxidation of the luciferin. A value proportional to the concentration of active luciferin initially present could be obtained by extrapolating back to zero time. The concentrations of active luciferin remaining after various time intervals were then expressed as percentages of the initial concentration determined in this way.

Experiments at any given temperature were ordinarily done in duplicate and showed very good agreement.

EXPERIMENTAL RESULTS

As already mentioned, Chase ('48) had found, and Johnson, Eyring and Polissar ('54) had deduced, that the non-luminescent oxidation of *Cypridina* luciferin was not as rapid a process as had been assumed by Chase and Lorenz ('45). For this reason the effect on the oxidation of luciferin of temperatures in the range from 26° to 46°C was studied in the present experiments.

As might have been expected, the actual rate of oxidation of luciferin in phosphate buffer of pH 6.8 was affected by the kind of luciferin preparation, although not markedly. Loss of active luciferin by oxidation was found to be about twice as fast in the case of luciferin purified by Tsuji's ('55) method as with a relatively crude methanol extract of lipid-free *Cypridina* powder. Also, the lower the luciferin concentration initially present, the faster was its loss by oxidation. Furthermore, if air was bubbled through the solution as it stood in the constant temperature bath the loss of active luciferin was more rapid than if it was not so aerated.

However, under none of the conditions tested was the rate of non-luminescent oxidation sufficiently fast to fit the mechanism postulated by Chase and Lorenz ('45). This is illustrated in figure 3. Here the curve which passes through the black dots is theoretical, calculated from a simple first order equation, while the dots are experimentally determined points and represent the per cent of active luciferin remaining during exposure, in pH 6.8 phosphate buffer, to a temperature of 31°C. The reaction is clearly first order and it is apparent that the loss of active luciferin by non-luminescent oxidation is fairly fast under these conditions. That it is not nearly fast enough, however, to account for the earlier interpretation by Chase and Lorenz

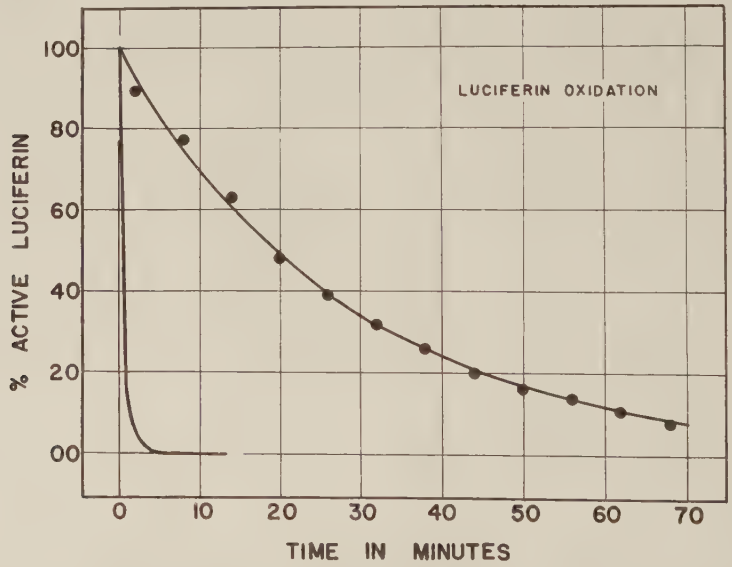

Fig. 3 The black dots indicate the percentage of active (unoxidized) luciferin remaining after standing in pH 6.8 phosphate buffer at 31°C. The curve drawn through the dots was calculated from the equation for a first order reaction. The steep curve at the left also represents the oxidation of luciferin but was obtained from the data of figure 1, as described in the text. It is evident that the two curves represent very different rates.

45) is shown by the other curve, to the ft, in figure 3. The latter is a calculated ırve representing the rate at which the on-luminescent oxidation of luciferin ıould proceed in order to fit into the ıechanism proposed by Chase and Lor-ız.

This curve was obtained in the follow-ıg way. In the case of the curve in figure which represents the course of the pre-ımed luminescent reaction at 30°C, it appens that the equation used by Chase nd Lorenz yields almost identical values or k_1 and k_2, the first order reaction rate onstants of the luminescent reaction and f the assumed non-luminescent oxidation f luciferin, respectively. Therefore, at 0° the curve representing the hypotheti-al non-luminescent oxidation of luciferin hould be practically the same as that ac-ually shown in figure 1 for the process of ight emission catalyzed by the enzyme, uciferase.

The left-hand, steeper curve in figure 3 s, then, simply the 30° curve of figure 1 lotted in terms of the ordinates of figure 3, nd represents the course which the non-uminescent oxidation of luciferin should ollow in order to fit into the scheme postu-ated by Chase and Lorenz. It is evident hat, although the actual non-luminescent xidation of *Cypridina* luciferin is by no neans a slow process, it is not nearly fast nough to account for the differences in otal light emitted by the luminescent re-ıction at different temperatures, as shown n figure 1.

Furthermore, it did not prove possible by such treatments as varying the con-entration of luciferin, using different luci-ferin preparations, or bubbling air through he solution, to increase the rate of non-uminescent oxidation of the luciferin sufficiently at pH 6.8 and 31° to fit the postulated scheme.

With the thought that possibly lucifer-ase, or some associated impurity, catalyzes he non-luminescent as well as the lumines-ent oxidation of luciferin, some experi-ments were done in which we added to uciferin solutions luciferase which had been inactivated by heat.[3] These did not show a significantly greater rate of loss of uciferin than did the controls. The luci-ferase was, of course, no longer in its native condition after such treatment, presumably, so that only a positive result would have been significant.

Temperature and the non-luminescent oxidation of luciferin

As stated above, although the non-luminescent oxidation of luciferin is not sufficiently rapid at 30°C to account for the effect of temperature on the total light emitted in the luminescent reaction, it is still markedly affected by temperature. Of the numerous experiments which were done over the temperature range from 26° to 46°C, a typical series will be described to illustrate the effect.

Figure 4 shows a family of curves representing the course of the non-luminescent oxidation of luciferin at 26°, 31°, 36°, and 40°C. The luciferin was a methanol extract of lipid-free *Cypridina* powder. The points are experimental but the curves themselves are theoretical, calculated from the equation for a first order reaction. The insert in figure 4 shows an Arrhenius plot of the relative reaction velocity constants at the 4 temperatures and yields an experimental activation energy of about 14,000 calories per mole. The corresponding value obtained by Chase and Lorenz ('45), on the assumption that differences in total light of the luminescent reaction at various temperatures reflected different rates of non-luminescent oxidation of luciferin, was 25,000 calories per mole. This is further indication that the effect of heat on total light emitted by the luminescent reaction does not involve the non-luminescent oxidation of the luciferin.

As mentioned above, the rate of loss of active luciferin by spontaneous, non-luminescent oxidation in phosphate buffer of pH 6.8 varied somewhat depending on the concentration of the luciferin and its method of preparation. For example, a 10-fold dilution of the active luciferin initially present resulted in a two to three-fold

[3] It is very difficult to inactivate this enzyme completely in this way. However, by autoclaving at 120°C for two hours all but a very small fraction of its activity was destroyed, as indicated by the fact that only extremely dim light could be seen with the dark-adapted eye when the heated enzyme was added to a large amount of active luciferin.

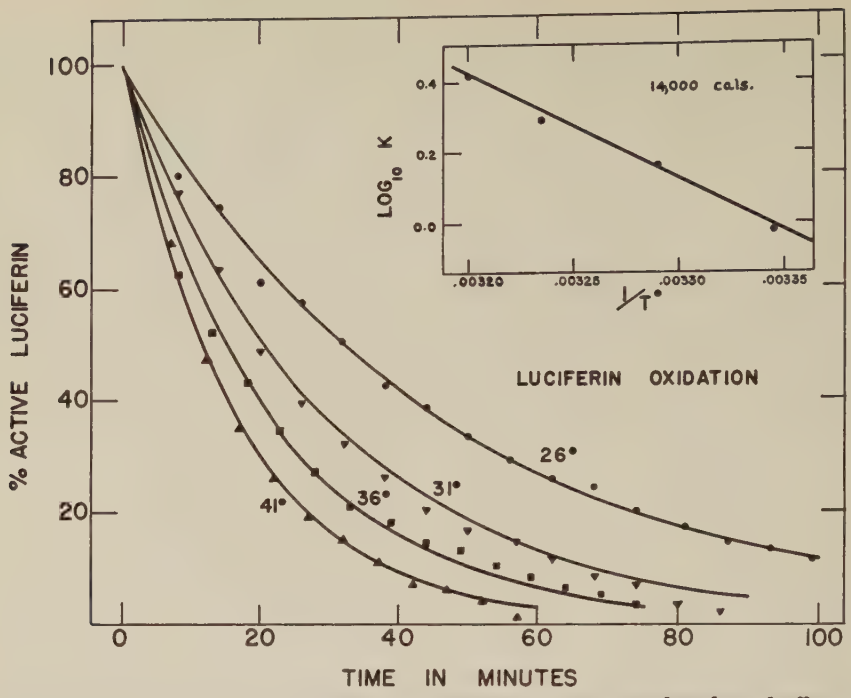

Fig. 4 Rate of loss of active luciferin on standing, in pH 6.8 phosphate buffer, at the temperatures indicated. The curves are theoretical, calculated from the equation for a simple first order reaction. The insert graph shows a plot of the logarithms of the relative first order velocity constants of the 4 curves against the reciprocal of the absolute temperature, and gives an experimental energy of activation of about 14,000 calories per mole.

increase in the rate of loss. Luciferin extracted and purified by Tsuji's ('55) method was oxidized about twice as fast as was a relatively crude methanol extract of the lipid-free *Cypridina* powder, although this effect may have been due merely to a lower concentration of luciferin in the more purified preparation.

Effect of pH

Anderson ('36) remarked that the stability of *Cypridina* luciferin against nonluminescent oxidation was less at pH 8 than at pH 5, and relatively great in dilute acid or alkali. No supporting data were given and it was therefore thought desirable to study the effect of pH on this process in more detail.

The experiments were done at 31°C, the procedure being the same as already described except that the pH was varied. Phosphate buffers, 0.2 M, made with Na_2HPO_4 and KH_2PO_4 were used for the pH's 5.3, 6.8, and 8.0. The stability of luciferin solutions in 0.1 N acid solution is so well known as to make its study unnecessary in the present experiments. The stated relative stability in dilute alkali, on the other hand, seemed so extraordinary that it was investigated in some detail, with and without phosphate ion being present. For these last experiments the mixture in which the luciferin was allowed to oxidize was either 0.18 N or 1.1 N NaOH, with or without 0.2 M K_3PO_4.

When removing a sample of such a mixture to determine the amount of active luciferin remaining, an amount of hydrochloric acid was added just sufficient to neutralize the free base in the sample and phosphate buffer was added simultaneously so that the sample finally contained NaCl and had a pH of 6.8. The luciferase, also in pH 6.8 phosphate buffer, was then added to catalyze the light production by the active luciferin present.

Figure 5 shows the results of experiments of this kind. As the curves show,

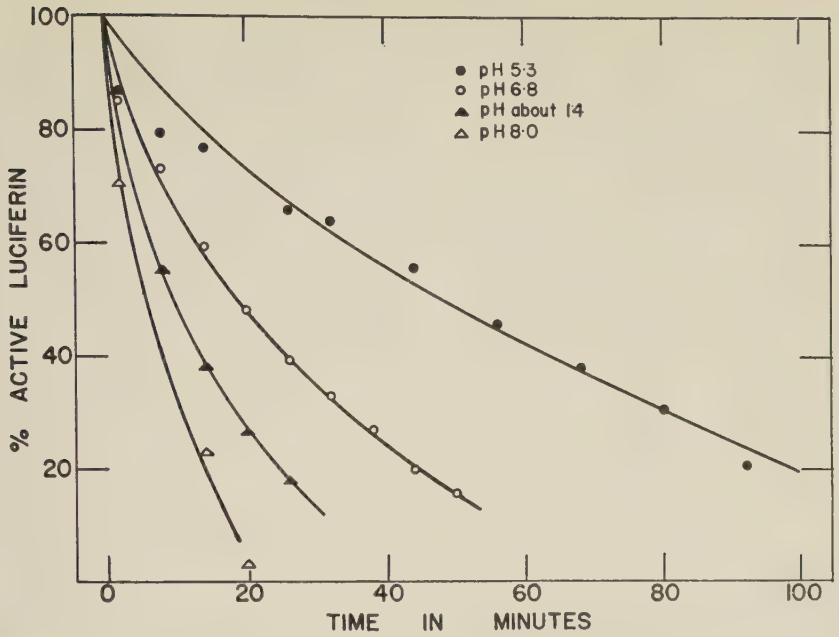

Fig. 5 Rate of loss of active luciferin on standing at 31°C, at the pH's indicated. Somewhere between pH 8 and pH 14 a condition of minimum stability against oxidation evidently exists.

ciferin is most stable against non-lumiescent oxidation at pH 5.3 (of the pH's udied), less so at pH 6.8, and still less so pH 8. In the dilute NaOH, on the other nd, the luciferin is *more* stable than at H 8, whether or not phosphate ion be esent. The curve labelled, "pH about ," actually represents the results obtained 0.18 or 1.1 N NaOH, with or without 2 M phosphate, although slightly greater ability was actually observed in 1.1 N aOH than in the lower concentration.

DISCUSSION AND CONCLUSIONS

The experimental work which has been one to date on the isolated luminescent action of luciferase and luciferin from *ypridina* shows certain apparent inconstencies which require consideration.

First, as was mentioned previously, differences in the total amount of light obtainable from the luciferin-luciferase reaction at different temperatures and at different hydrogen ion concentrations can be ttributed only in small part, if at all, to he loss of active luciferin through nonminescent oxidation. Indeed, at least in he absence of luciferase, the non-luminescent oxidation of luciferin is much too slow a process to account for differences in total light observed in the experiments illustrated in figure 1. A quenching effect of heat upon the luciferin or upon the luciferin-luciferase complex, or whatever the actual light emitter is, must presumably be almost wholly responsible for the differences in the total light emitted when luciferin and luciferase react.

The possibility that the non-luminescent oxidation of *Cypridina* luciferin proceeds differently in the presence of luciferase than in its absence must, however, be considered for if the oxidation were much more rapid with the enzyme present, the equation of Chase and Lorenz could still apply. There is, however, one piece of experimental evidence which definitely does not favor this. When the luminescent reaction is run with a constant initial concentration of luciferin but different concentrations of luciferase, the first order reaction rate constant varies with the luciferase concentration as would be expected. However, if the luciferase, or something associated with it, also catalyzed the non-luminescent oxidation of luciferin, the to-

tal light obtained from the luminescent reaction should vary inversely with the concentration of luciferase present. This is not found experimentally. The total amount of light produced is approximately the same, regardless of the concentration of the enzyme. It can probably be concluded, therefore, that the non-luminescent oxidation of luciferin proceeds at the same rate in the presence of luciferase as in its absence. The experiments described above, in which the addition of heat-inactivated luciferase to luciferin solutions had no effect on the rate of non-luminescent oxidation of luciferin, are also consistent with this observation.

Anderson ('37) observed that the total amount of light emitted in the reaction of luciferin and luciferase under constant conditions was greatly influenced by certain ions. Most caused a quenching effect but the chloride ion produced an increase in total light. He also reported (Anderson, '33) that the amount of light obtainable from luciferin was dependent upon the hydrogen ion concentration, and Chase ('48) studied this dependence in some detail. Here there was relatively little, if any, contribution by the non-luminescent oxidation of luciferin. Practically the entire effect could be attributed to presumed differences in the efficiency of the emitting system.

It seems most likely, then, that the differences in total light obtained from the luciferin-luciferase reaction at different temperatures, as exemplified by the curves of figure 1, represent essentially a quenching effect of heat on the light-emitting molecule or complex. Indeed, Johnson, Eyring and Polissar ('54, pp. 153–159) have fitted these same experimental data fairly well with an equation representing changes in the efficiency of the light-emitting system as a function of temperature.

The temperature optimum of about 23°C for luciferase activity obtained mathematically through the interpretation by Chase and Lorenz ('45) may therefore be too low by about 4° and the enzyme may be more active at 35° than they calculated.

Another puzzling characteristic of the luciferase-luciferin system is the fact that the reaction rate constant of the luminescent reaction, presumably representing the activity of the luciferase, is maximal a 27°C (assuming no contribution by th non-luminescent oxidation of the luciferin and rather low at 35°C (Chase and Lo enz, '45). Yet if the inactivation of luc ferase by heat be measured in the absenc of luciferin (Chase, '50), practically n loss of activity of the enzyme is observe at temperatures below 40°! Clearly, th effect of heat must be very different o the isolated enzyme than upon the enzym substrate complex, unless the first orde reaction rate constant of the luminescen reaction represents something else in ad dition to the activity of the luciferase There is, of course, a basic difference i the experimental methods of measurin the luciferase activity in the studies b Chase and Lorenz ('45) and by Chas ('50). In the former the first order re action rate constant (presumably indicat ing activity of the enzyme) was determine at the temperature whose inactivating ef fect was being examined. In the latte experiments, on the other hand, the luci ferase alone was subjected to the elevate temperatures and samples were periodic ally removed, and the inactivation assaye by adding luciferin and measuring th luminescent reaction at room tempera ture. Any rapidly reversible inactivation of luciferase, such as that observed b Chase ('46), would not have shown itsel in the experiments by Chase ('50) on the isolated enzyme.

The final explanation for apparent dis crepancies such as these may have t await the availability of really pure *Cypri dina* luciferin and luciferase. In all the work which has been done on this lumines cent reaction, the presence of impurities has again and again affected the experi mental results.

SUMMARY

The effects of hydrogen ion concentra tion and of temperatures from 26° to 46°C were studied for the non-luminescent oxi dation of luciferin, extracted by two me thods from *Cypridina hilgendorfii*.

It was found that, at pH 6.8 and 30°C the oxidation of luciferin in the absence of luciferase was half complete in about 20 minutes. This reaction, although relatively rapid, is therefore not sufficiently fast to

count for the differences observed in al light produced at different tempera- es by the luminescent reaction of luci- in and luciferase. The assumption of ase and Lorenz ('45) that two concur- nt first order reactions, one the lumines- nt reaction itself and the other a non- minescent oxidation of luciferin, are re- onsible for differences in total light pro- ced at different temperatures, is not sup- rted. The temperature optimum for this *vitro* luminescent reaction is, conse- ently, at about 27° rather than 23°C, d differences in total light produced at ferent temperatures are presumably used by quenching effects on the light- itting compound or system.

Over the temperature range from 26° 46°C the non-luminescent oxidation of iferin, in pH 6.8 phosphate buffer, var- in such a way as to yield an experi- ntal activation energy of about 14,000 ories per mole.

Not more than a two to three-fold change absolute rate of the non-luminescent dation of luciferin was produced by such atments as diluting the luciferin, using iferin isolated from *Cypridina* by two tirely different methods, or bubbling air rough the solution.

The addition of heat-inactivated luci- ase to luciferin solutions of pH 6.8 at °C had no measurable effect on the e of the non-luminescent oxidation of iferin.

The luciferin was less stable against n-luminescent oxidation in phosphate ffer of pH 6.8 than pH 5.3 and still less stable in pH 8 phosphate buffer. In 0.18 or 1.1 N sodium hydroxide, whether or not phosphate were present, the luciferin was more stable than in pH 8 phosphate buffer.

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COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

The Effects of Different Salt Concentrations on the Affinity of Hemoglobin for Oxygen[1]

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Fifty years ago Barcroft and Camis ('09) and Barcroft and Roberts ('09) reported that sodium chloride and potassium chloride increased the affinity of hemoglobin for oxygen. Kono ('31) stated that neutral salts have no effect on oxygen dissociation in low concentrations, but in high concentrations these salts depress dissociation to the same degree when isotonic solutions are used. Green and Talbot ('33) showed that the dissociation curve of horse hemoglobin shifted symmetrically to the right with increased sodium chloride concentrations as oxy-hemoglobin affinity was decreased. According to Sidwell et al. ('38), the addition of various salts (NaCl, KCl, Na_2SO_4, $Na_3C_6H_5O_7$, NaH_2PO_4, and Na_2HPO_4) in different concentrations to dialyzed human hemoglobin inhibited the oxygenation of hemoglobin. They showed that the inhibiting effect increased with the nature of the salts in the order listed. However, experimental evidence is lacking which shows the effect of various buffered salinities on oxy-hemoglobin affinity as related to the study of oxygen dissociation curves.

MATERIALS AND METHODS

Approximately 60 ml of human blood was withdrawn by antecubital venipuncture into a heparinized collecting bottle. Immediately 4–5 ml of blood was placed in rubber-stoppered pieces of tygon tubing, and stored at 0°C. Later 1.0 ml of blood was removed from a thawed and well-mixed sample tube, laked immediately in 9.0 ml of distilled water, and centrifuged for 20 minutes at 5000 r.p.m. Five milliliters of the supernatant was pipetted into a 50-ml glass-stoppered, graduated cylinder, diluted to 50 ml with Sorensen's phosphate buffer (6.8 pH), mixed, and placed in an ice bath until ready for use. It was this solution, representing a 1: 100 dilution of hemoglobin in buffer solution, on which the oxygen dissociation curve was determined. This was done by transferring 5.0 ml of the buffered hemoglobin solution to special tonometers, and equilibrating at different oxygen tensions in a constant temperature water bath (37.0 ± 0.03°C). The Hall Spectro-Comparator and the procedures described by Hall ('34) and ('35) were used, although the techniques were a modification of those described by Foreman ('54).

The essence of these techniques is a microspectroscopical comparison of the equilibrated sample with known standards of both reduced and oxygenated hemoglobin. Different values were determined for the per cent saturation of oxy-hemoglobin at different oxygen tensions, and plotted on coordinate paper described by Brown and Hill ('22). The oxygen dissociation curve was plotted by extrapolation of these data. In establishing the curve, each plot value represented the mean value of three sample determinations, a fresh sample being used for each equilibration. Dissociation curves were determined for hemoglobin solutions made-up in different molarities of Sorensen's phosphate buffer ranging from 0.001 M to 1 M concentration.

RESULTS

Figure 1 represents the relation of the oxygen tension of half-saturation of hemo-

[1] This work was done during the summer of 1957 under the direction of Dr. F. G. Hall, Chairman, Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina.

[2] Research Fellow of the American Physiological Society, and sponsored by the National Institutes of Health, Bethesda, Maryland.

lobin in millimeters of Hg to different alinity concentrations of Sorensen's phos- hate buffer solution at 37.0 ± 0.03°C and H 6.8. The $T_{1/2\ sat}$ point plots represent he mean of three dissociation curves de- ermined at each molar concentration vhen the concentration of oxy-hemoglobin quals that of reduced hemoglobin. Refer- nce to the figure shows that a $T_{1/2\ sat}$ mean alue for human hemoglobin of 23.0 mm Ig is maintained in the salinity range of .0133 M to 0.100 M concentration. The esults also indicate that an increase or a ecrease in salinity beyond the range stated bove inhibits oxygenation of hemoglobin.

DISCUSSION OF RESULTS

After the discovery of carbhemoglobin y Henriques ('28), Stadie and O'Brien '37) pointed out that although carbon lioxide depresses the affinity of hemo- globin for oxygen by forming carbamino compounds of oxy-hemoglobin and reduced hemoglobin, hemoglobin at its iso-electric point (pH 6.8) does not form carbamate. Consequently, in this study hemoglobin was buffered at iso-electric pH.

The concentration of buffered saline solution of hemoglobin which is used for the determination of oxygen dissociation curves is dependent on the physiological isosmotic value determined for the blood of each species. In mammals, an isotonic NaCl solution of 0.90% which is considered to be physiologically isosmotic, corresponds to a molar concentration of 0.154. Hence, in order to increase endosmosis so that hemoglobin would be liberated, it is necessary that hemoglobin be buffered in salinities which assures red blood corpuscle lysis. But the quantitative limits of saline molarity effects on oxy-hemoglobin affinity as related to the study of oxygen dissociation curves have not been established.

It can be seen in figure 1 that the $T_{1/2\ sat}$ values determined at each molarity are

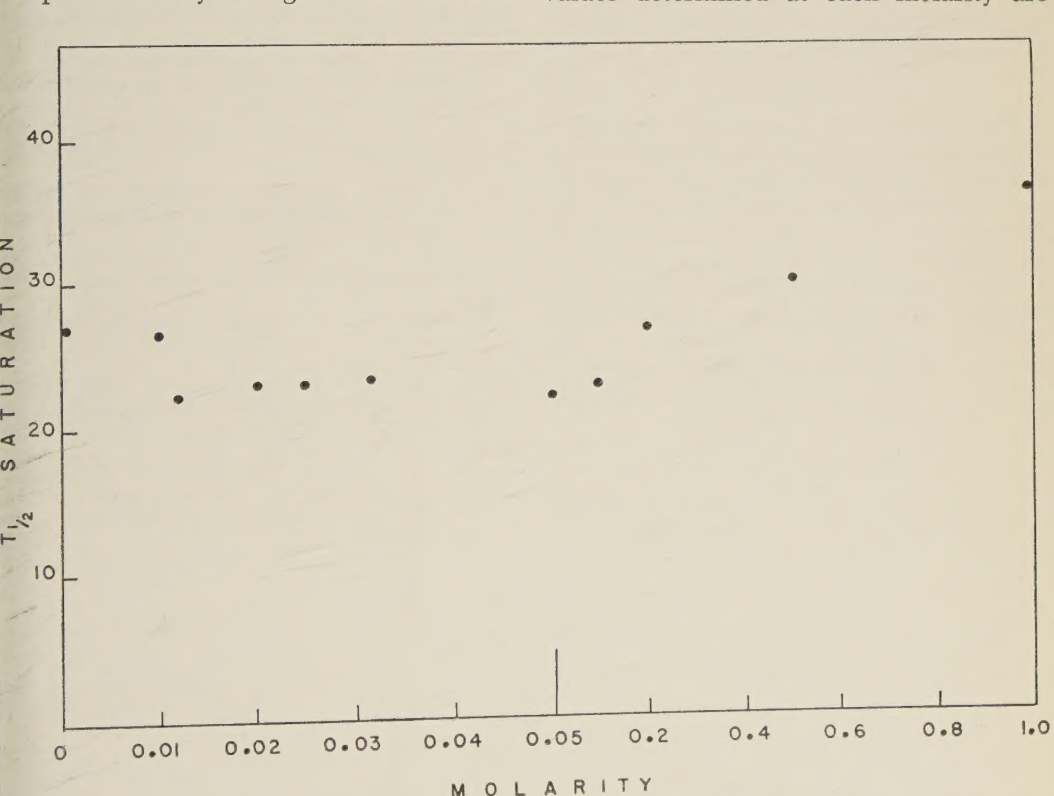

Fig. 1 The effect of different molarities of Sorensen's phosphate buffer at pH 6.8 on the oxygen ension of hemoglobin (when Hb equals HbO_2) equilibrated at 37.0 ± 0.03°C.

of the same magnitude in the range from 0.013 M to 0.100 M. The mean value of these 18 determinations for $T_{1/2\ sat}$ of hemoglobin is 23.0 mm Hg. In 23 oxygen dissociation curves, using a buffered hemoglobin solution of 0.066 M, Hall ('36) reported a $T_{1/2sat}$ figure of 23.5 mm Hg. Foreman ('54), using a 0.033 M buffered hemoglobin solution, reported a $T_{1/2sat}$ value of 21.7 mm Hg. Therefore, it appears that valid oxygen dissociation curves can be determined on dilute hemoglobin solutions when buffered in salinities between 0.013 M and 0.100 M.

Although not intended for investigation here, it can be seen in the figure that the $T_{1/2\ sat}$ value increases (indicating a shift to the right of the oxygen dissociation curve) when the salt concentration is either increased or decreased. Sidwell et al. ('38) remark that in some instances the inhibiting action of increased salt concentrations on hemoglobin oxygenation may not be due to an ionic strength effect, but probably to a combination of anions with hemoglobin.

SUMMARY

The Hall Spectro-Comparator was used to determine 33 oxygen dissociation curves of human hemoglobin in 11 different salinities ranging from 0.001 M to 1.0 M, and buffered at pH 6.8 with equilibration of each sample carried out at 37.0 ± 0.03°C. A $T_{1/2\ sat}$ value of 23.0 mm Hg was determined in a buffered salinity range from 0.013 M to 0.100 M. Increasing or decreasing the salinity concentration beyond the range stated above depressed the affinity of hemoglobin for oxygen.

ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. F. G. Hall for suggesting this problem, for his personal encouragement, and providing the necessary laboratory equipment and space; The American Physiological Society and the National Institutes of Health for financial aid during the tenure of the Research Fellowship; Dr. Ray G. Daggs, Executive Secretary, The American Physiological Society and Dr. W. R. Amberson, Chairman, Department of Physiology, University of Maryland for personal advice.

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